SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES USING LEAF EXTRACT OF *Azadirachta indica*

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By

TAMASA PANIGRAHI

411LS2058

Under The Supervision of

Dr. SUMAN JHA

DEPARTMENT OF LIFE SCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY

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I dedicate my dissertation work to my beloved parents and my sister.

A special feeling of gratitude to my loving parents whose words of encouragement and push for tenacity ring in my ears. My sister has never left my side and is very special.

DECLARATION

I Tamasa Panigrahi hereby declare that, this project report entitled **"Synthesis and Characterization of Silver Nanoparticles using leaf extract of *Azadirachta indica*"**, submitted by me, under the guidance of Dr. Suman Jha, Associate Professor, N.I.T., Rourkela is my own and has not been submitted to any other University or Institute or published earlier.

Rourkela

Date: Tamasa Panigrahi

Dr. Suman Jha

Associate Professor

Department of Life Science

National Institute of Technology, Rourkela, Orissa, India

CERTIFICATE

This is to certify that the thesis entitled **"Synthesis and Characterization of Silver Nanoparticles using leaf extract of *Azadirachta indica*"** by **Tamasa Panigrahi (411LS2058)**, submitted to the National Institute of Technology, Rourkela for the Degree of Master of Science is a record of bonafide research work, carried out by her in the Department of Life Science under my supervision. I believe that the thesis fulfils part of the requirements for the award of Master of Science. The results embodied in the thesis have not been submitted for the award of any other degree

Dr. Suman Jha

Department of Life Science

NIT Rourkela

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Tamasa panigrahi

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LIST OF ABBREVIATIONS

UV-Vis	**Ultra Violet Visible Spectroscopy**
SEM	**Scanning Electron Microscope**
DLS	**Dynamic Light Scattering**
FTIR	**Fourier Transform Infra Red Spectroscope**
XRD	**X-Ray Diffraction**
SPR	**Surface Plasmon Resonance**
PBS	**Phosphate Buffered Saline**
µL	**Micro Litter**
rpm	**Rotation Per Minute**
Hrs	**Hours**
KBr	**Potassium Bromide**
nm	**Nano-Meter**
$AgNO_3$	**Silver Nitrate**
Ag_NPs	**Silver Nanoparticles**
min	**Minutes**
mV	**Mili volt**

ABSTRACT:

In recent science Nanotechnology is a burning field for the researchers. Nanotechnology deals with the Nanoparticles having a size of 1-100 nm in one dimension used significantly concerning medical chemistry, atomic physics, and all other known fields. Nanoparticles are used immensely due to its small size, orientation, physical properties, which are reportedly shown to change the performance of any other material which is in contact with these tiny particles. These particles can be prepared easily by different chemical, physical, and biological approaches. But the biological approach is the most emerging approach of preparation, because, this method is easier than the other methods, ecofriendly and less time consuming. The Green synthesis was done by using the aqueous solution of ***Azadirachta indica*** leaf extract and $AgNO_3$. Silver was of a particular interest for this process due to its evocative physical and chemical properties. A fixed ratio of plant extract to metal ion was prepared and the color change was observed which proved the formation of nanoparticles. The nanoparticles were characterized by UV-vis Spectrophotometer, FTIR, DLS, Zeta Analysis, XRD, and SEM. The nanoparticles were found have the size ranges from 160-180 nm.

Key Words: Nanotechnology, Nanoparticles, Green Synthesis

INTRODUCTION

Due to swift industrialization and urbanization, our environment is undergo huge smash up and a large amount of perilous and superfluous chemical, gases or substances are released, and so now it is our need to learn about the secrets that are present in the Nature and its products which leads to the growth of advancements in the synthesis processes of nanoparticles. Nanotechnology applications are highly suitable for biological molecules, because of their exclusive properties. The biological molecules undergo highly controlled assembly for making them suitable for the metal nanoparticle synthesis which was found to be reliable and eco friendly [1] . The synthesis of metal and semiconductor nanoparticles is a vast area of research due to its potential applications which was implemented in the development of novel technologies [2]. The field of nanotechnology is one of the upcoming areas of research in the modern field of material science. Nanoparticle show completely new or improved properties, such as size, distribution and morphology of the particles etc. Novel applications of nanoparticles and nanomaterials are emerging rapidly on various fields [3].

Metal nanoparticles have a high specific surface area and a high fraction of surface atoms. Because of the unique physicochemical characteristics of nanoparticles, including catalytic activity, optical properties, electronic properties, antibacterial properties, and magnetic properties [4-7] they are gaining the interest of scientist for their novel methods of synthesis. Over the past few years, the synthesis of metal nanoparticles is an important topic of research in modern material science. Nano-crystalline silver particles have been found tremendous applications in the fields of high sensitivity biomolecular detection, diagnostics, antimicrobials, therapeutics, catalysis and micro-electronics. However, there is still need for economic commercially viable as well as environmentally clean synthesis route to synthesize the silver nanoparticles. Silver is well known for possessing an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes [8]. In medicines, silver and silver nanoparticles have a ample application including skin ointments and creams containing silver to prevent infection of burns and open wounds [9], medical devices and implants prepared with silver-impregnated polymers [10]. In textile industry, silver-embedded fabrics are now used in sporting equipment [11].

Nanoparticles can be synthesized using various approaches including chemical, physical, and biological. Although chemical method of synthesis requires short period of time for synthesis of large quantity of nanoparticles, this method requires capping agents for size stabilization of the nanoparticles. Chemicals used for nanoparticles synthesis and stabilization are toxic and lead to non-ecofriendly byproducts. The need for environmental non-toxic synthetic protocols for nanoparticles synthesis leads to the developing interest in biological approaches which are free from the use of toxic chemicals as byproducts. Thus, there is an increasing demand for ‘‘green nanotechnology” [12]. Many biological approaches for both extracellular and intracellular nanoparticles synthesis have been reported till date using microorganisms including bacteria, fungi and plants [13, 14].

Plants provide a better platform for nanoparticles synthesis as they are free from toxic chemicals as well as provide natural capping agents. Moreover, use of plant extracts also reduces the cost of microorganisms isolation and culture media enhancing the cost competitive feasibility over nanoparticles synthesis by microorganisms [12].

Sometimes the synthesis of nanoparticles using various plants and their extracts can be advantageous over other biological synthesis processes which involve the very complex procedures of maintaining microbial cultures [15, 16]. Many such experiments have already been started such as the synthesis of various metal nanoparticles using fungi like *Fusarium oxysporum* [17], *Penicillium* sp. [18] and using some bacteria such as *Bacillus subtilis* etc. [19, 20]. But, synthesis of nanoparticles using plant extracts is the most adopted method of green, eco-friendly production of nanoparticles and also has a special advantage that the plants are widely distributed, easily available, much safer to handle and act as a source of several metabolites [21]. There has also been several experiments performed on the synthesis of silver nanoparticles using medicinal plants such as *Oryza sativa*, *Helianthus annus*, *Saccharum officinarum*, *Sorghum bicolour*, *Zea mays*, *Basella alba*, *Aloe vera Capsicum annuum*, *Magnolia kobus*, *Medicago sativa* (Alfalfa), C*inamomum camphora* and *Geranium* sp. in the field of pharmaceutical applications and biological industries. Besides, green synthesis of silver nanoparticles using a methanolic extract of *Eucalyptus hybrida* was also investigated [22].

In the recent days, silver nanoparticles have been synthesized from the naturally occurring sources and their products like green tea (*Camellia sinensis)*, Neem (*Azadirachta indica)* , leguminous shrub(*Sesbania drummondii*) , various leaf broth, natural rubber, starch , *Aloe vera* plant extract , lemongrass leaves extract, etc. [23]. With respect to the microbes, the silver nanoparticles get attached to the cell wall, thereby disturbing the permeability of cell wall and cellular respiration. The nanoparticles may also penetrate deep inside the cell wall, thus causing cellular damage by interacting with phosphorus and sulfur containing compounds, such as DNA and protein, present inside the cell. The bacteriocidal properties of silver nanoparticles are due to the release of silver ions from the particles, which confers the antimicrobial activity [24]. Besides, the potency of the antibacterial effects corresponds to the size of the nanoparticle. The smaller particles have higher antibacterial activities due to the equivalent silver mass content. With respect to the clinical applications of nanoparticle, microorganisms including diatoms, fungi, bacteria and yeast producing inorganic materials through biological synthesis either intra or extracellularly made nanoparticles more biocompatible [25].

LITERATURE REVIEW

Nanotechnology is an important field of modern research dealing with design, synthesis, and manipulation of particles structure ranging from approximately 1-100 nm in one dimension. Remarkable growth in this up-and-coming technology has opened novel fundamental and applied frontiers, including the synthesis of nanoscale materials and exploration or utilization of their exotic physicochemical and optoelectronic properties. Nanotechnology is rapidly gaining importance in a number of areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, reorography, single electron transistors, light emitters, nonlinear optical devices, and photoelectrochemical applications [26, 27]. Nanomaterials are seen as solution to many technological and environmental challenges in the field of solar energy conversion, catalysis, medicine, and water treatment. In the context of global efforts to reduce hazardous waste, the continuously increasing demand of nanomaterials must be accompanied by green synthesis methods.

Nanotechnology is fundamentally changing the way in which materials are synthesized and devices are fabricated. Incorporation of nanoscale building blocks into functional assemblies and further into multifunctional devices can be achieved through a “bottom-up approach”. Research on the synthesis of nanosized material is of great interest because of their unique properties like optoelectronic, magnetic, and mechanical, which differs from bulk [28].

NANOPARTICLES:

The term “nanoparticles” is used to describe a particle with size in the range of 1nm-100nm, at least in one of the three possible dimensions. In this size range, the physical, chemical and biological properties of the nanoparticles changes in fundamental ways from the properties of both individual atoms/molecules and of the corresponding bulk materials. Nanoparticles can be made of materials of diverse chemical nature, the most common being metals, metal oxides, silicates, non-oxide ceramics, polymers, organics, carbon and biomolecules. Nanoparticles exist in several different morphologies such as spheres, cylinders, platelets, tubes etc. Generally the nanoparticles are designed with surface modifications tailored to meet the needs of specific applications they are going to be used for. The enormous diversity of the nanoparticles **(Figure. 1)** arising from their wide chemical nature, shape and morphologies, the medium in which the particles are present, the state of dispersion of the particles and most importantly, the numerous possible surface modifications the nanoparticles can be subjected to make this an important active field of science now-a-days.

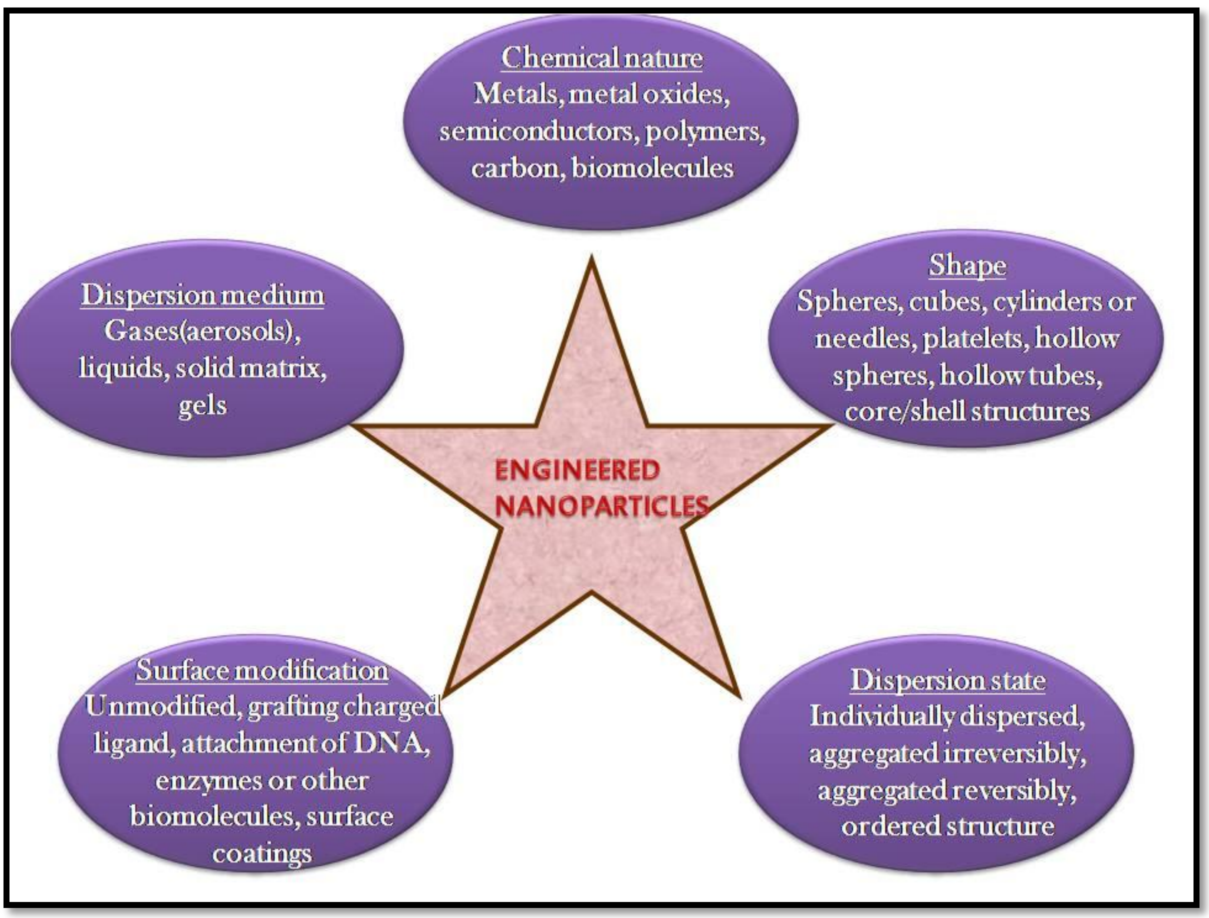

Fig. 1: Various features contributing to the diversity of engineered nanoparticles. The same chemical can generate a wide variety of nanoparticles.

TYPES OF NANOPARTICLES:

Nanoparticles can be broadly grouped into two, namely, organic nanoparticles which include carbon nanoparticles (fullerenes) while, some of the inorganic nanoparticles include magnetic nanoparticles, noble metal nanoparticles (like gold and silver) and semi-conductor nanoparticles (like titanium oxide and zinc oxide). There is a growing interest in inorganic nanoparticles i.e. of noble metal nanoparticles (Gold and silver) as they provide superior material properties with functional versatility. Due to their size features and advantages over available chemical imaging drug agents and drugs, inorganic particles have been examined as potential tools for medical imaging as well as for treating diseases. Inorganic nonmaterial have been widely used for cellular delivery due to their versatile features like wide availability, rich functionality, good compatibility, and capability of targeted drug delivery and controlled release of drugs [29].

SILVER NANOPARTICLES:

Silver nanoparticles are of interest because of the unique properties (*e.g.,* size and shape depending optical, electrical, and magnetic properties) which can be incorporated into antimicrobial applications, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products, and electronic components. Several physical and chemical methods have been used for synthesizing and stabilizing silver nanoparticles [30, 31]. The most popular chemical approaches, including chemical reduction using a variety of organic and inorganic reducing agents, electrochemical techniques, physicochemical reduction, and radiolysis are widely used for the synthesis of silver nanoparticles. Recently, nanoparticle synthesis is among the most interesting scientific areas of inquiry, and there is growing attention to produce nanoparticles using environmentally friendly methods (green chemistry). Green synthesis approaches include mixed-valence polyoxometalates, polysaccharides, Tollens, biological, and irradiation method which have advantages over conventional methods involving chemical agents associated with environmental toxicity. This chapter presents an overview of silver nanoparticle preparation by physical, chemical, and green synthesis approaches. The aim of this chapter is, therefore, to reflect on the current state and future prospects, especially the potentials and limitations of the above mentioned techniques for industries. Moreover, we discuss the applications of silver nanoparticles and their incorporation into other materials, the mechanistic aspects of the antimicrobial effects of silver nanoparticles.

METHODS FOR NANOPARTICLE SYNTHESIS:

Physical approaches:

Most important physical approaches include evaporation-condensation and laser ablation. Various metal nanoparticles such as silver, gold, lead sulfide, cadmium sulfide, and fullerene have previously been synthesized using the evaporation-condensation method. The absence of solvent contamination in the prepared thin films and the uniformity of nanoparticles distribution are the advantages of physical approaches in comparison with chemical processes. [32, 33]. It was demonstrated that silver nanoparticles could be synthesized via a small ceramic heater with a local heating source [34]. The evaporated vapor can cool at a suitable rapid rate, because the temperature gradient in the vicinity of the heater surface is very steep in comparison with that of a tube furnace. This makes possible the formation of small nanoparticles in high concentration. This physical method can be useful as a nanoparticle generator for long-term experiments for inhalation toxicity studies, and as a calibration device for nanoparticle measurement equipment [34]. Silver nanoparticles could be synthesized by laser ablation of metallic bulk materials in solution [35-39]. The ablation efficiency and the characteristics of produced nanosilver particles depend upon many factors such as the wavelength of the laser impinging the metallic target, the duration of the laser pulses (in the femto-, pico- and nanosecond regime), the laser fluence, the ablation time duration and the effective liquid medium, with or without the presence of surfactants [40-43]. One important advantage of laser ablation technique compared to other methods for production of metal colloids is the absence of chemical reagents in solutions. Therefore, pure and uncontaminated metal colloids for further applications can be prepared by this technique [44].

Chemical approaches:

The most common approach for synthesis of silver nanoparticles is chemical reduction by organic and inorganic reducing agents. In general, different reducing agents such as sodium citrate, ascorbate, sodium borohydride (NaBH4), elemental hydrogen, polyol process, Tollens reagent, N, N-dimethylformamide (DMF), and poly (ethylene glycol)-block copolymers are used for reduction of silver ions (Ag^+) in aqueous or non-aqueous solutions. The aforementioned reducing agents reduce silver ions (Ag^+) and lead to the formation of metallic silver (Ag^0), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to formation of metallic colloidal silver particles [46-48]. It is important to use protective agents to stabilize dispersive nanoparticles during the course of metal nanoparticle preparation, and protect the nanoparticles that can be absorbed on or bind onto nanoparticle surfaces, avoiding their agglomeration [49]. The presence of surfactants comprising functionalities (*e.g.,* thiols, amines, acids, and alcohols) for interactions with particle surfaces can stabilize particle growth, and protect particles from sedimentation, agglomeration, or losing their surface properties.

Recently, a simple one-step process, Tollens method, has been used for the synthesis of silver nanoparticles with a controlled size. In the modified Tollens procedure, silver ions are reduced by saccharides in the presence of ammonia, yielding silver nanoparticle films (50-200 nm), silver hydrosols (20-50 nm) and silver nanoparticles of different shapes [52].

Biological approaches:

In recent years, the development of efficient green chemistry methods employing natural reducing, capping, and stabilizing agents to prepare silver nanoparticles with desired morphology and size have become a major focus of researchers. Biological methods can be used to synthesize silver nanoparticles without the use of any harsh, toxic and expensive chemical substances [53, 54]. The bioreduction of metal ions by combinations of biomolecules found in the extracts of certain organisms (*e.g.,* enzymes/proteins, amino acids, polysaccharides, and vitamins) is environmentally benign, yet chemically complex.

Many studies have reported successful synthesis of silver nanoparticle using organisms (microorganisms and biological systems) [55, 56].

Synthesis of silver nanoparticles by bacteria:

The first evidence of bacteria synthesizing silver nanoparticles was established using the *Pseudomonas stutzeri* AG259 strain that was isolated from silver mine [57]. There are some microorganisms that can survive metal ion concentrations and can also grow under those conditions, and this phenomenon is due to their resistance to that metal. The mechanisms involved in the resistance are efflux systems, alteration of solubility and toxicity via reduction or oxidation, biosorption, bioaccumulation, extracellular complex formation or precipitation of metals, and lack of specific metal transport systems [58]. There is also another aspect that though these organisms can grow at lower concentrations, their exposure to higher concentrations of metal ions can induce toxicity.

The most widely accepted mechanism of silver biosynthesis is the presence of the nitrate reductase enzyme. The enzyme converts nitrate into nitrite. In in vitro synthesis of silver using bacteria, the presence of alpha-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) - dependent nitrate reductase would remove the downstream processing step that is required in other cases. [59].

Synthesis of silver nanoparticles by fungi:

When in comparison with bacteria, fungi can produce larger amounts of nanoparticles because they can secrete larger amounts of proteins which directly translate to higher productivity of nanoparticles [61]. The mechanism of silver nanoparticle production by fungi is said to follow the following steps: trapping of Ag+ ions at the surface of the fungal cells and the subsequent reduction of the silver ions by the enzymes present in the fungal system [62]. The extracellular enzymes like naphthoquinones and anthraquinones are said to facilitate the reduction. Considering the example of F. oxysporum, it is believed that the NADPH-dependent nitrate reductase and a shuttle quinine extracellular process are responsible for nanoparticle formation [63]. Though the exact mechanism involved in silver nanoparticle production by fungi is not fully deciphered, it is believed that the abovementioned phenomenon is responsible for the process. A major drawback of using microbes to synthesize silver nanoparticles is that it is a very slow process when in comparison with plant extracts. Hence, the use of plant extracts to synthesize silver nanoparticles becomes an option that is feasible.

Synthesis of silver nanoparticles by plants:

The major advantage of using plant extracts for silver nanoparticle synthesis is that they are easily available, safe, and nontoxic in most cases, have a broad variety of metabolites that can aid in the reduction of silver ions, and are quicker than microbes in the synthesis. The main mechanism considered for the process is plant-assisted reduction due to phytochemicals. The main phytochemicals involved are terpenoids, flavones, ketones, aldehydes, amides, and carboxylic acids. Flavones, organic acids, and quinones are water-soluble phytochemicals that are responsible for the immediate reduction of the ions. Studies have revealed that xerophytes contain emodin, an anthraquinone that undergoes tautomerization, leading to the formation of the silver nanoparticles. In the case of mesophytes, it was found that they contain three types of benzoquinones: cyperoquinone, dietchequinone, and remirin. It was suggested that the phytochemicals are involved directly in the reduction of the ions and formation of silver nanoparticles [64].

NEED FOR GREEN SYNTHESIS:

Biosynthesis of nanoparticles is a kind of bottom up approach where the main reaction occurring is reduction/oxidation. The need for biosynthesis of nanoparticles rose as the physical and chemical processes were costly. Often, chemical synthesis method leads to presence of some of the toxic chemical absorbed on the surface that may have adverse effect in the medical applications [65]. This is not an issue when it comes to biosynthesized nanoparticles via green synthesis route [66]. So, in the search of cheaper pathways for nanoparticles synthesis, scientist used microbial enzymes and plant extracts (phytochemicals). With their antioxidant or reducing properties they are usually responsible for the reduction of metal compounds into their respective nanoparticles. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals .

NANOSILVER:

One of the substances used in nanoformulation is silver (nanosilver). Due to its antimicrobial properties, silver has also been incorporated in filters to purify drinking water and clean swimming pool water. To generate nanosilver, metallic silver has been engineered into ultrafine particles by several methods; include spark discharging, electrochemical reduction, solution irradiation and cryo- chemical synthesis [67]. Nano-silver particles are mostly smaller than 100 nm and consist of about 20-15,000 silver atoms [67]. In addition, nanostructures can be produced as tubes, wires, multifactes or films. At the nano-scale, the silver particles exhibit deviating physico-chemical properties (like pH dependent partitioning to solid and dissolved particulate matters) and biological activites compared with the regular metal [68].This is due to the higher surface area per mass, allowing a larger amount of atoms to interact with their surroundings. Due to the properties of silver at the nanoscale, nanosilver is nowadays used in an increasing number of consumer and medical products. Because, silver is a soft white lustrous element, an important use of silver nanoparticles is to give a products a silver finish. Still, the remarkably strong antimicrobial activity is the major direction for development of nano-silver products. Examples are food packaging materials and food supplements, odour-resistant textiles, electronics, household appliances, cosmetics and medical advices, water disinfectants and room sprays.

WHY SILVER ?

Silver is one of the basic element that makes up our planet. It is a rare, but naturally occurring element, slightly harder than gold and very ductile and malleable. Pure silver has the highest electrical and thermal conductivity of all metals and has the lowest contact resistance. Silver can be present in four different oxidation states: Ag^0, Ag^{2+}, Ag^{3+}. The former two are the most abundant ones, the latter are unstable in the aquatic environment [69]. Metallic silver itself is insoluble in water, but metallic salts such as $AgNO_3$ and Silver chloride are soluble in water (WHO,2002).Metallic silver is used for the surgical prosthesis and splints, fungicides and coinage. Soluble silver compounds such as silver slats, have been used in treating mental illness, epilepsy, nicotine addition, gastroenteritis and infectious diseases including syphilis and gonorrhea [69]. Although acute toxicity of silver in the environment is dependent on the availability of free silver ions, investigations have shown that these concentrations of Ag^+ ions are too low to lead toxicity (WHO, 2002). Metallic silver appears to pose minimal risk to health, whereas soluble silver compounds are more readily absorbed and have the potential to produce adverse effects [70]. The wide variety of uses of silver allows exposure through various routes of entry into the body. Ingestion is the primary route for entry for silver compounds and colloidal silver proteins. Dietary intake of silver is estimated at 70-90μg/day. Since silver in any form is not thought to be toxic to the immune , cardiovascular, nervous or reproductive system an it is not considered to be carcinogenic [71], therefore silver is relatively non-toxic [72]. Silver demand will likely to rise as silver find new uses, particularly in textiles, plastics and medical industries, changing the pattern of silver emission as these technologies and products diffuse through the global economy [69].

ACTION OF SILVER NANOPARTICLES ON MICROBES:

The exact mechanism which silver nanoparticles employ to cause antimicrobial effect is not clearly known and is a debated topic. There are however various theories on the action of silver nanoparticles on microbes to cause the microbicidal effect. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of ‘pits’ on the cell surface, and there is accumulation of the nanoparticles on the cell surface [73]. The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die. There have been electron spin resonance spectroscopy studies that suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death [74, 75]. It has also been proposed that there can be release of silver ions by the nanoparticles [76], and these ions can interact with the thiol groups of many vital enzymes and inactivate them [77]. The bacterial cells in contact with silver take in silver ions, which inhibit several functions in the cell and damage the cells. Then, there is the generation of reactive oxygen species, which are produced possibly through the inhibition of a respiratory enzyme by silver ions and attack the cell itself. Silver is a soft acid, and there is a natural tendency of an acid to react with a base, in this case, a soft acid to react with a soft base . The cells are majorly made up of sulfur and phosphorus which are soft bases. The action of these nanoparticles on the cell can cause the reaction to take place and subsequently lead to cell death. Another fact is that the DNA has sulfur and phosphorus as its major components; the nanoparticles can act on these soft bases and destroy the DNA which would definitely lead to cell death [78]. The interaction of the silver nanoparticles with the sulfur and phosphorus of the DNA can lead to problems in the DNA replication of the bacteria and thus terminate the microbes. It has also been found that the nanoparticles can modulate the signal transduction in bacteria. It is a wellestablished fact that phosphorylation of protein substrates in bacteria influences bacterial signal transduction. Dephosphorylation is noted only in the tyrosine residues of gram-negative bacteria. The phosphotyrosine profile of bacterial peptides is altered by the nanoparticles. It was found that the nanoparticles dephosphorylate the peptide substrates on tyrosine residues, which leads to signal transduction inhibition and thus the stoppage of growth. It is however necessary to understand that further research is required on the topic to thoroughly establish the claims [79] **(Figure 2)**.

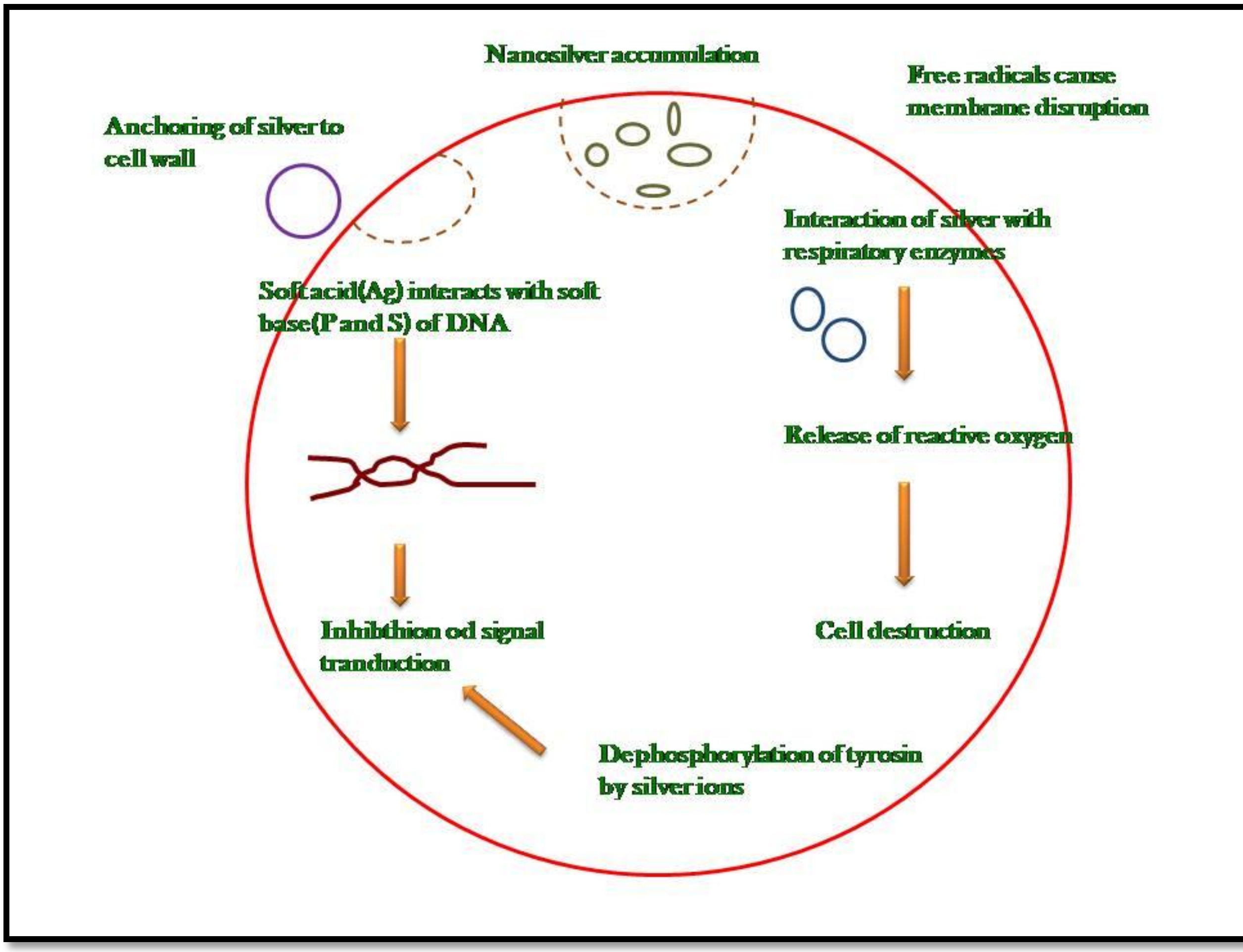

Fig. 2: Various modes of action of silver nanoparticles on bacteria.

APPLICATIONS OF SILVER NANOPARTICLES AND THEIR INCORPORATION INTO OTHER MATERIALS:

Nanoparticles are of great interest due to their extremely small size and large surface to volume ratio, which lead to both chemical and physical differences in their properties compared to bulk of the same chemical composition, such as mechanical, biological and sterical properties, catalytic activity, thermal and electrical conductivity, optical absorption and melting point [80]. Therefore, designing and production of materials with novel applications can be resulted by controlling shape and size at nanometer scale. Nanoparticles exhibit size and shape-dependent properties which are of interest for applications ranging from biosensing and catalysts to optics, antimicrobial activity, computer transistors, electrometers, chemical sensors, and wireless electronic logic and memory schemes. These particles also have many applications in different fields such as medical imaging, nano-composites, filters, drug delivery, and hyperthermia of tumors [81, 82]. Silver nanoparticles have drawn the attention of researchers because of their extensive applications in areas such as integrated circuits [22, 83], sensors [83], biolabelling ,filters , antimicrobial deodorant fibres [84], cell electrodes [85], low-cost paper batteries (silver nano-wires) [86] and antimicrobials [87, 88]. Silver nanoparticles have been used extensively as antimicrobial agents in health industry, food storage, textile coatings and a number of environmental applications. Antimicrobial properties of silver nanoparticles caused the use of these nano-metals in different fields of medicine, various industries, animal husbandry, packaging, accessories, cosmetics, health and military. [87, 88]. For instance, it was shown that silver nanoparticles mainly in the range of 1-10 nm attached to the surface of *E. coli* cell membrane, and disturbed its proper function such as respiration and permeability **(Figure. 3)**.

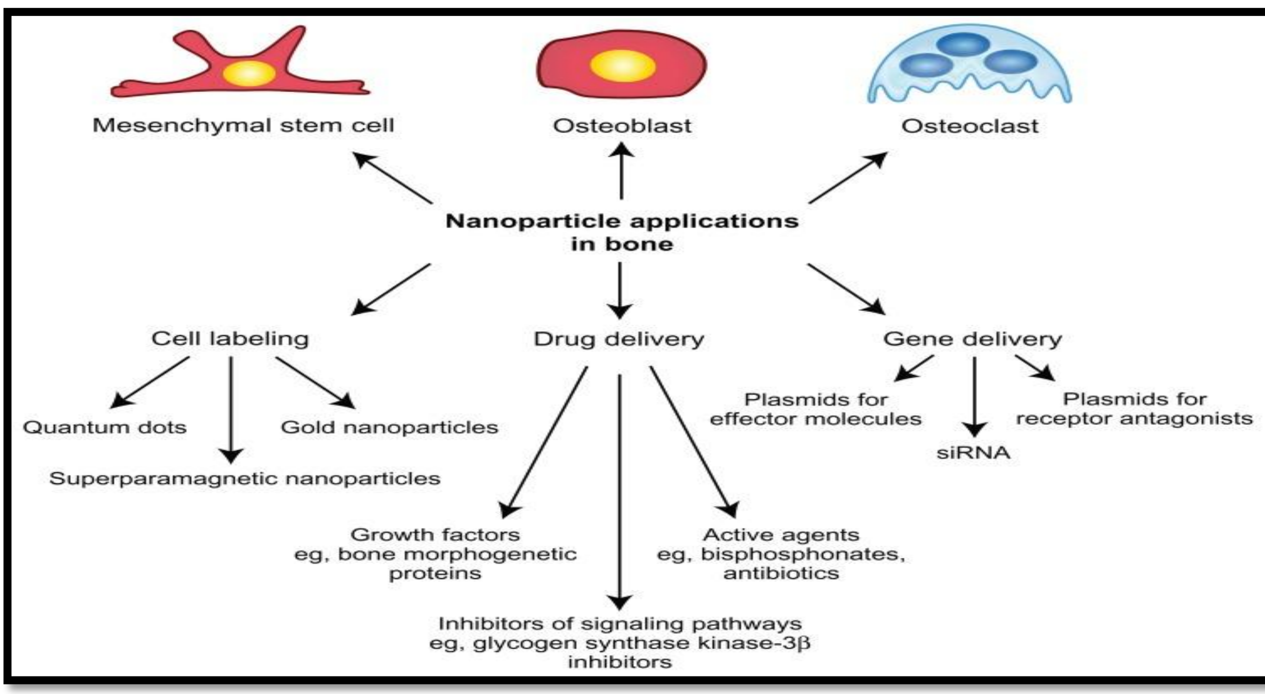

Fig 3: Overview of nanoparticle applications [98]

In general, therapeutic effects of silver particles (in suspension form) depend on important aspects, including particle size (surface area and energy), particle shape (catalytic activity), particle concentration (therapeutic index) and particle charge (oligodynamic quality) [90]. Mechanisms of antimicrobial effects of silver nanoparticles are still not fully understood, but several studies have revealed that silver nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, which leads to denaturation of protein and finally cell death **(Table 1)**. Fluorescent bacteria were used to study antibacterial effects of silver nanoparticles. Green fluorescent proteins were adapted to these investigations [34]. It was found that silver nanoparticles attached to sulfur-containing proteins of bacteria, and caused death. Moreover, fluorescent measurements of cell-free supernatants showed the effect of silver nanoparticles on recombination of bacteria. The attachment of silver ions or nanoparticles to the cell wall caused accumulation of envelope protein precursors resulting in immediate dissipation of the proton motive force [99]. Catalytic mechanism of silver nanoparticle composites and their damage to the cell by interaction with phosphorous- and sulfur-containing compounds such as DNA have been also investigated [100]. Furthermore, silver nanoparticles exhibited destabilization of the outer membrane and rupture of the plasma membrane, thereby causing depletion of intracellular ATP [68]. Another mechanism involves the association of silver with oxygen and its reaction with sulfhydryl groups on the cell wall to form R-S-S-R bonds, thereby blocking respiration and causing cell death **(Table 2)** [101].

Table 1: Applications of silver nanoparticles in pharmaceutics, medicine, and dentistry

• **Applications of silver nanoparticles in pharmaceutics, medicine, and dentistry**	
Pharmaceutics & Medicines	• **Treatment of dermatitis; inhibition of HIV-1 replication** • **Treatment of ulcerative colitis & acne** • **Antimicrobial effects against infectious organisms** • **Remote laser light-induced opening of microcapsules** • **Silver/dendrimer nanocomposite for cell labeling** • **Molecular imaging of cancer cells** • **Enhanced Raman Scattering (SERS) spectroscopy** • **Detection of viral structures (SERS & Silver nanorods)** • **Coating of hospital textile (surgical gowns, face mask)** • **Additive in bone cement** • **Implantable material using clay-layers with starch-stabilized Ag NPs** • **Orthopedic stocking** • **Hydrogel for wound dressing**
Dentistry	• **Additive in polymerizable dental materials Patent** • **Silver-loaded SiO_2 nanocomposite resin filler (Dental resin composite)** • **Polyethylene tubes filled with fibrin sponge embedded with Ag NPs dispersion**

Table 2: Mechanisms of antibacterial effects of silver nanoparticles

Mechanisms of Antibacterial Effects of Ag NPs
Cell death due to uncoupling of oxidative phosphorylation
Cell death due to induction of free radical formation
Interference with respiratory chain at Cyt C level
Interference with components of microbial ETS
Interactions with protein thiol groups & membrane bound enzymes
Interaction with phosphorous- and sulfur-containing compounds such as DNA

TOXICITY OF SILVER NANOPARTICLES:

The unique physical and chemical properties of silver nanoparticles make them excellent candidates for a number of day-to-day activities, and also the antimicrobial and anti-inflammatory properties make them excellent candidates for many purposes in the medical field. However, there are studies and reports that suggest that nanosilver can allegedly cause adverse effects on humans as well as the environment. It is estimated that tonnes of silver are released into the environment from industrial wastes, and it is believed that the toxicity of silver in the environment is majorly due to free silver ions in the aqueous phase. The adverse effects of these free silver ions on humans and all living beings include permanent bluish-gray discoloration of the skin (argyria) or the eyes (argyrosis), and exposure to soluble silver compounds may produce toxic effects like liver and kidney damage; eye, skin, respiratory, and intestinal tract irritations; and untoward changes in blood cells [131]. Since the beginning of the twenty-first century, nanosilver has been gaining popularity and is now being used in almost every field, most importantly the medical field. However, there have been reports of how nanosilver cannot discriminate between different strains of bacteria and can hence destroy microbes beneficial to the ecology [132]. There are only very few studies conducted to assess the toxicity of nanosilver. In one study, in vitro toxicity assay of silver nanoparticles in rat liver cells has shown that even low-level exposure to silver nanoparticles resulted in oxidative stress and impaired mitochondrial function [133]. Silver nanoparticles also proved to be toxic to in vitro mouse germ line stem cells as they impaired mitochondrial function and caused leakage through the cell membranes. Nanosilver aggregates are said to be more cytotoxic than asbestos [134]. There is evidence that shows that silver ions cause changes in the permeability of the cell membrane to potassium and sodium ions at concentrations that do not even limit sodium, potassium, ATP, or mitochondrial activity [135]. The literature also proves that nanosilver can induce toxic effects on the proliferation and cytokine expression by peripheral blood mononuclear cells [136]. Nanosilver is also known to show severe toxic effects on the male reproductive system. Research shows that nanosilver can cross the blood-testes barrier and be deposited in the testes where they adversely affect the sperm cells [137]. Even commercially available silver-based dressings have been proved to have cytotoxic effects on various experimental models [138]. In vivo studies on the oral toxicity of nanosilver on rats have indicated that the target organ in mouse for the nanosilver was the liver. It was also found from histopathological studies that there was a higher incidence of bile duct hyperplasia, with or without necrosis, fibrosis, and pigmentation in the study animals [139]. Studies have also suggested that there is release of silver when the nanoparticles are stored over a period of time. Hence, it has to be said that aged nanosilver is more toxic than new nanosilver [140]. Nanosilver with its antimicrobial activity can hinder the growth of many ‘friendly’ bacteria in the soil. By showing toxic effects on denitrifying bacteria, silver can disrupt the denitrification process, which involves the conversion of nitrates into nitrogen gas which is essential for the plants. Loss of environmental denitrification through reduction of plant productivity can lead to eutrophication of rivers, lakes, and marine ecosystems and destroy the ecosystem. Nanosilver also has toxic effects on aquatic animals because silver ions can interact with the gills of fish and inhibit basolateral Na+-K+-ATPase activity, which can in turn inhibit osmoregulation in the fish [141]. To understand the toxic potential nanosilver has on the freshwater environment, the Daphnia magna 48-h immobilization test was conducted, and the results showed that the silver nanoparticles have to be classified under ‘category acute 1’ as per the Globally Harmonized System of Classification and Labelling of Chemicals, suggesting that the release of nanosilver into the environment has to be carefully considered [142]. Though these studies tend to suggest that nanosilver can induce toxicity to living beings, it has to be understood that the studies on nanosilver toxicity were done in in vitro conditions which are drastically different from in vivo conditions and at quite high concentrations of nanosilver particles. Hence, it is imperative that more studies be carried out to assess the toxicity effect nanosilver has in vivo before a conclusion on its toxicity is reached.

MATERIALS AND METHODS

PREPARATION OF PLANT EXTRACT:

From *Azadirachta indica* (Neem) leaves:

Fresh leaves of ***Azadirachta indica***, **(Figure 4)** were collected from N.I.T. RKL campus, and washed several times with water to remove the dust particles and then sun dried to remove the residual moisture and grinded to form powder. Then plant extract was prepared by mixing 1% of plant extract with deionized water in a 250ml of (Borosil, India) conical flask. Then the solution was incubated for 30 min. and then subjected to centrifuge for 30 min. at room temperature with 5000 rpm. The supernatant was separated and filterd with **(mm filter paper pore size)** filter paper with the help of vaccume filter. Then the solution was used for the reduction of silver ions Ag^{+}) to silver nanoparticles (Ag^{o}).

Fig. 4: Picture of *Azadirachta indica* leaves

From *Acorus calamus* (Sweet flag) rhizome:

Dried rhizomes of Acorus calamus **(Figure 5)** were brought from Pondicherry and grinded to form powder. Then plant extract was prepared by mixing 1% of plant extract with deionized water in a 250ml of (Borosil, India) conical flask. Then the solution was incubated for 30 min. and then subjected to centrifuge for 30 min. at room temperature with 5000 rpm. The supernatant was separated and filterd with **(mm filter paper pore size)** filter paper with the help of vaccume filter. Then the solution was used for the reduction of silver ions (Ag^{+}) to silver nanoparticles (Ag^{o}).

Fig. 5: Picture of *Acorus calamus* rhizomes

Synthesis of Silver Nanoparticles:

Four concentration ratios of plant and metal ions were prepared (30:1, 60:1,120:1 & 240:1) by increasing the concentration of plant extract concentration in the solution. 0.17% of 1mM $AgNO_3$ metal ion was added in the prepared plant extract. Then the bioreduced aqueous component was used to measuring UV-Vis spectra of the solution.

CHARACTERIZATION OF SILVER NANOPARTICLES:

UV-Vis Analysis:

The optical property of Ag_NPs was determined by UV-Vis spectrophotometer (Perkin-Elmer,Lamda 35,Germany). After the addition of $AgNO_3$ to the plant extract, the spectras were taken in different time intervals up to 24Hrs. between 350 nm to 500 nm. Then the spectra was taken after 24Hrs. of $AgNO_3$ addition.

FTIR analysis:

The chemical composition of the synthesized silver nanoparticles was studied by using FTIR spectrometer **(**perkin-Elmer LS-55- Luminescence spectrometer**)**. The solutions were dried at 75^{o} C and the dried powders were characterized in the range 4000–400 cm^{-1} using KBr pellet method.

XRD Analysis:

The phase variety and grain size of synthesized Silver nanoparticles was determined by X-ray diffraction spectroscopy (Philips PAN analytical). The synthesized silver nanoparticles were studies with CUKα radiation at voltage of 30 kV and current of 20 MA with scan rate of 0.03^0/s. Different phases present in the synthesized samples were determined by X′ pert high score software with search and match facility. The particle size of the prepared samples were determined by using Scherrer′s equation as follows

$$D \approx \frac{0.9\lambda}{\beta \cos\theta}$$

Where D is the crystal size, λ is the wavelength of X-ray, Θ is the Braggs angle in radians and B is the full width at half maximum of the peak in radians.

SEM Analysis:

The morphological features of synthesized silver nanoparticles from neem plant extract were studied by Scanning Electron Microscope (JSM-6480 LV). After 24Hrs. of the addition of $AgNO_3$ the SEM slides were prepared by making a smear of the solutions on slides. A thin layer of platinum was coated to make the samples conductive. Then the samples were characterized in the SEM at an accelerating voltage of 20 KV

DLS & Zeta-Potential Analysis:

Dynamic light scattering (DLS) which is based on the laser diffraction method with multiple scattering techniques was employed to study the average particle size of silver nanoparticles. The prepared sample was dispersed in deionised water followed by ultra-sonication. Then solution was filtered and centrifuged for 15 min.at 25^o C with 5000 rpm and the supernatant was collected. The supernatant was diluted for 4 to 5 times and then the particle distribution in liquid was studied in a computer controlled particle size analyzer (ZETA sizer Nanoseries,Malvern instrument Nano Zs).

RESULTS AND DICUSSIONS

UV-Vis SPECTROPHOTOMETER ANALYSIS:

Reduction of silver ions into silver nanoparticles during exposure to plant extracts was observed as a result of the color change. The color change is due to the Surface Plasmon Resonance phenomenon. The metal nanoparticles have free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. The sharp bands of silver nanoparticles were observed around 421 nm in case of *Azadirachta indica* **Figure: 7, Figure:8, Figure:9, & Figure:10)** whereas the bands for *Acorus calamus* were observed around 384 nm **(Figure:6)**.From different literatures it was found that the silver nanoparticles show SPR peak at around 420 nm. From our studies we found the SPR peak for *Azadirachta indica* at 421 nm whereas for *Acorus calamus* it was at 384 nm. So we confirmed that *Azadirachta indica* leaf extract has more potential to reduce Ag ions into Ag nanoparticles than *Acorus calamus* extract, which lead us for further research on synthesis of silver nanoparticles from *Azadirachta indica* leaf extracts. The intensity of absorption peak increases with increasing time period. This characteristic color variation is due to the excitation of the SPR in the metal nanoparticles the insets to Figure: 7, Figure:8, Figure:9 & Figure:10 represent the plots of absorbance at λ_{max} (i.e., at 420 nm) versus time of reation. The reduction of the metal ions occurs fairly rapidly; more than 90% of reduction of Ag^+ ions is complete within 4 Hrs. after addition of the metal ions to the plant extract. The metal particles were observed to be stable in solution even 4 weeks after their synthesis. By stability, we mean that there was no observable variation in the optical properties of the nanoparticle solutions with time.

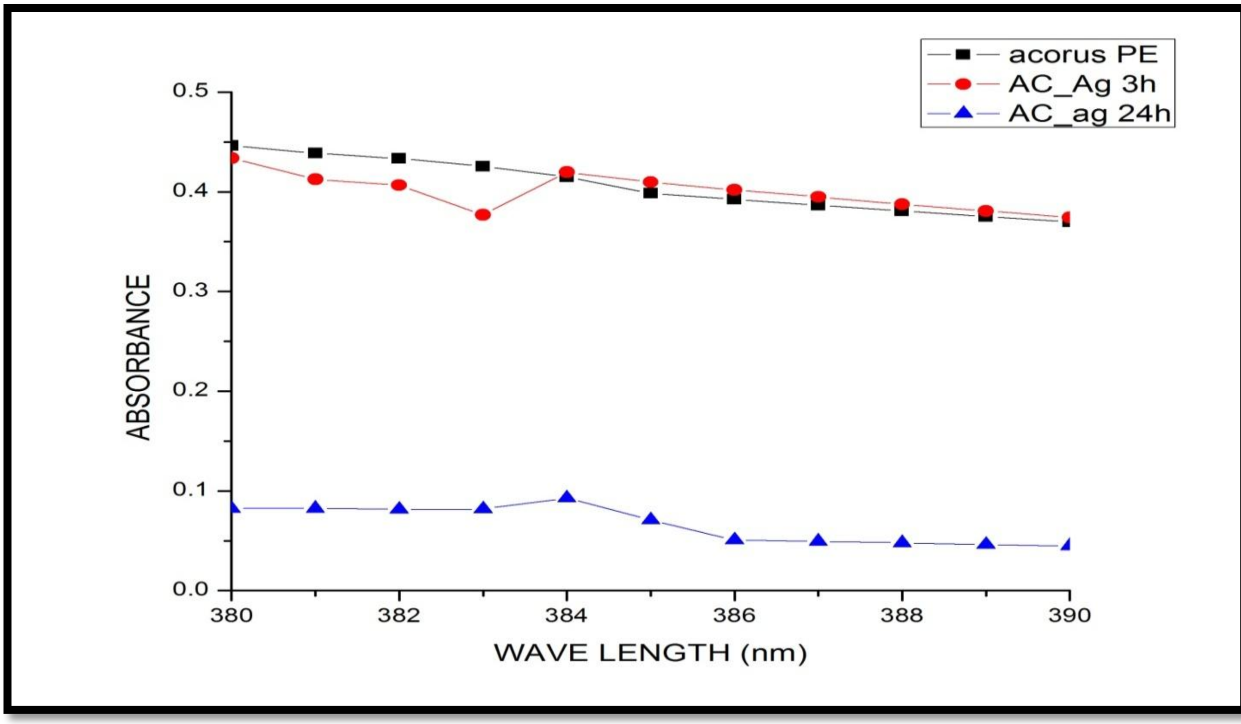

Fig.6: UV-vis spectra of *Acorus. calamus* at different time interval

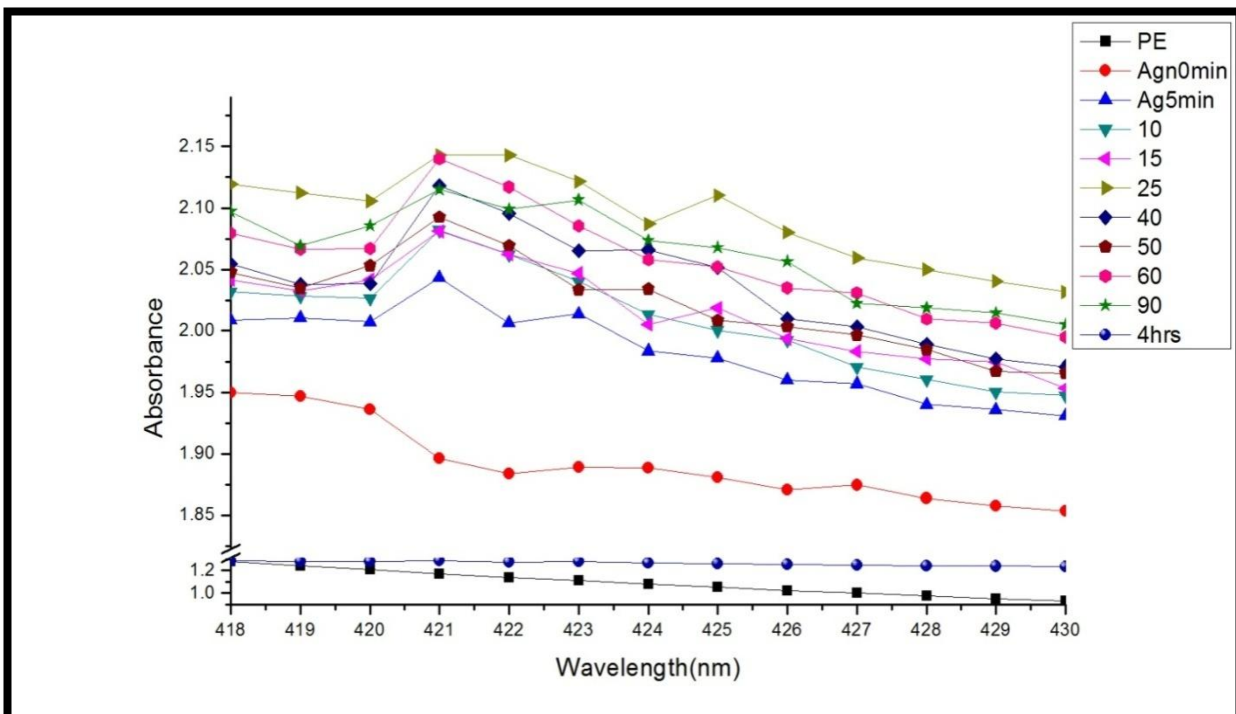

Fig.7: UV-vis spectra of *Azadirachta indica* 30:1 ratio at different time interval

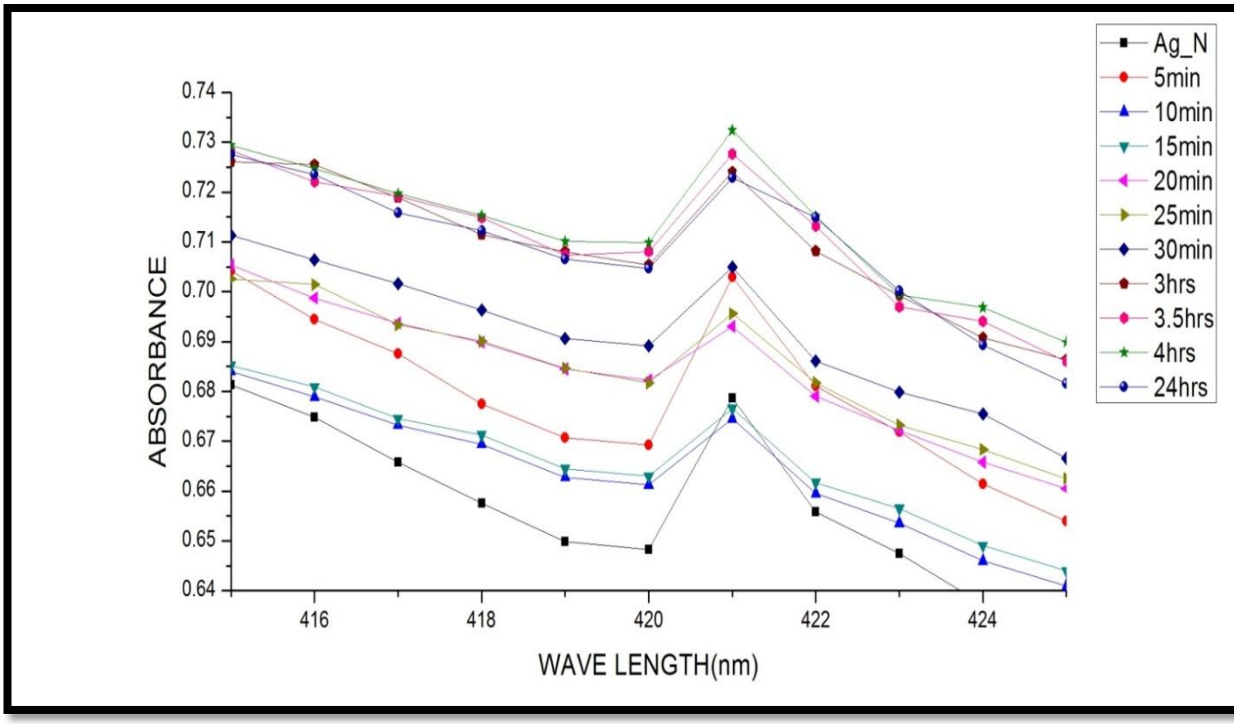

Fig.8: UV-vis spectra of *Azadirachta indica* 60:1 ratio at different time interval

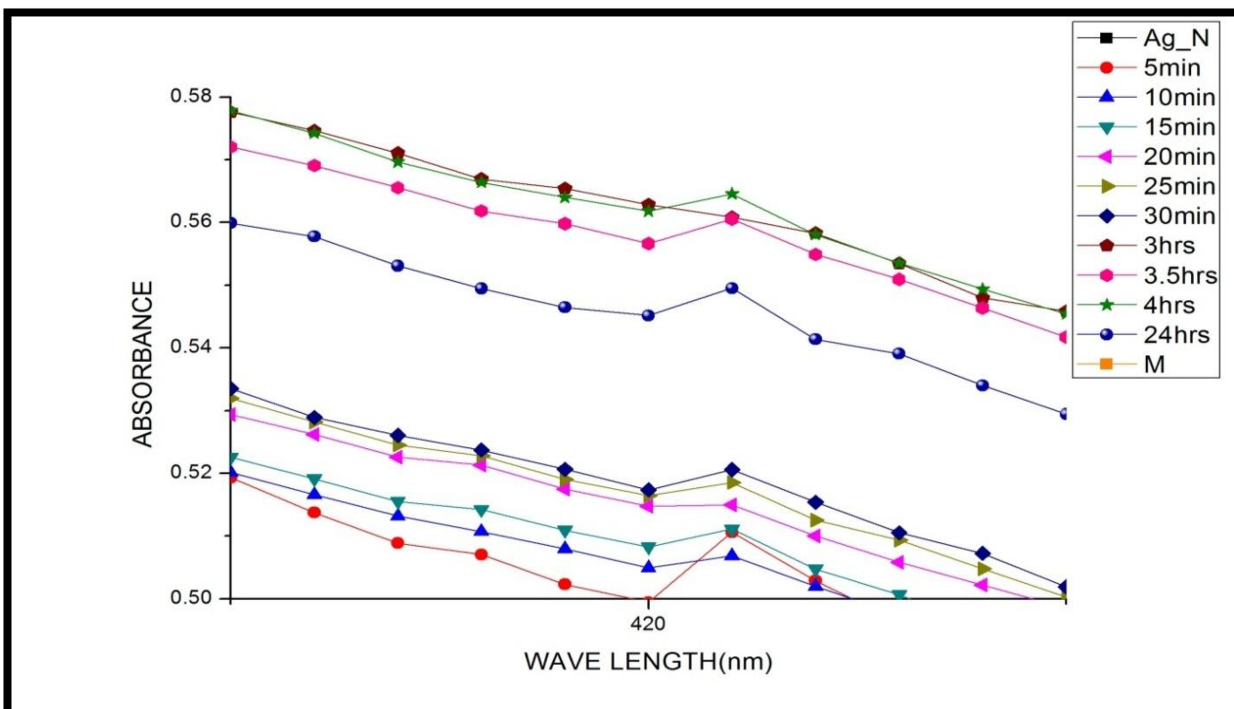

Fig.9: UV-vis spectra of *Azadirachta indica* 120:1 ratio at different time interval

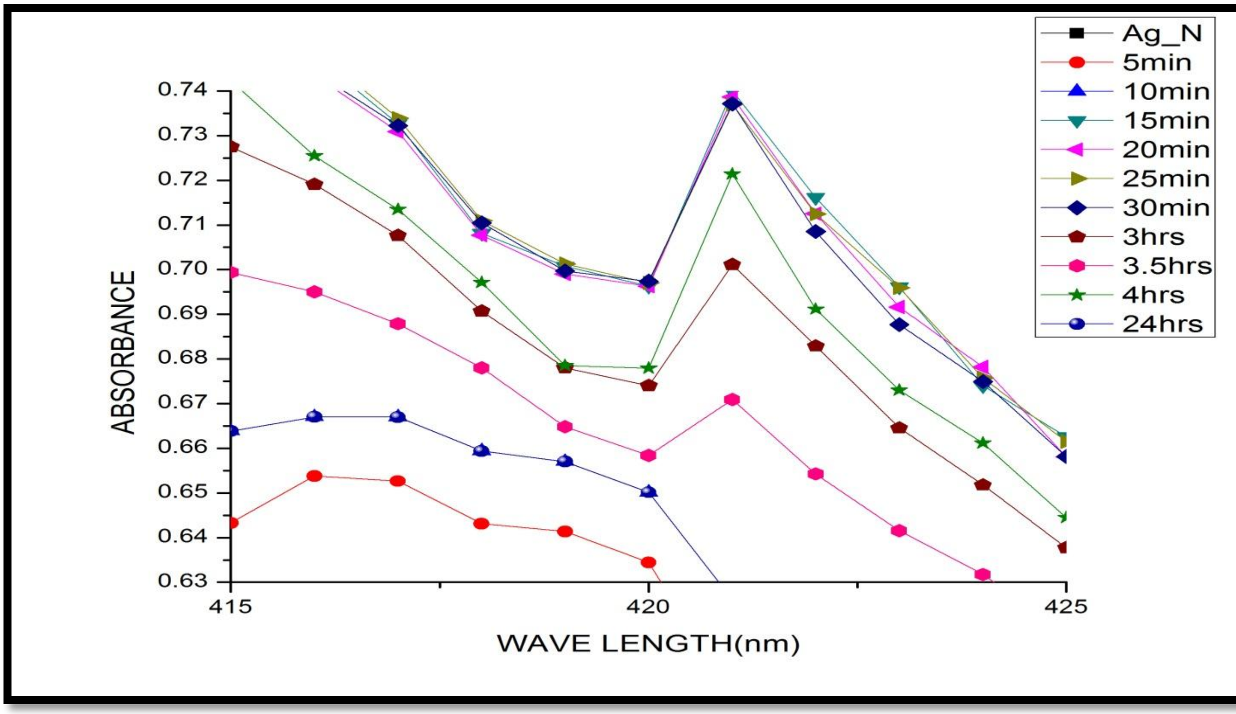

Fig.10: UV-vis spectra of *Azadirachta indica* 240:1 ratio at different time interval

On the behalf of UV-vis data it was cleared that *A. indica* reduces metal ions better than *A. calamus.* So the further characterizations were carried out with *A. indica*.

SEM ANALYSIS:

SEM provided further insight into the morphology and size details of the silver nanoparticles. Comparison of experimental results showed that the diameters of prepared nanoparticles in the solution have sizes of several µm in case of 30:1, 120:1 & 240:1 ratios where as in 60:1 ratio the size is of several nm. . **(Figure: 11, Figure: 12, Figure: 13 & Figure: 14)**. The size of the prepared nanoparticles was more than the size of nanoparticle which should be; i.e.; between 1-100 nm. The size was more than the desired size as a result of the proteins which were bound in the surface of the nanoparticles. The result showed that the particles were of spherical shape in case of 30:1, 60:1, and 120:1 ratios but sheet shape in case of 240:1 ratio. The shape varies due to the concentration increased in 240:1 ratio.

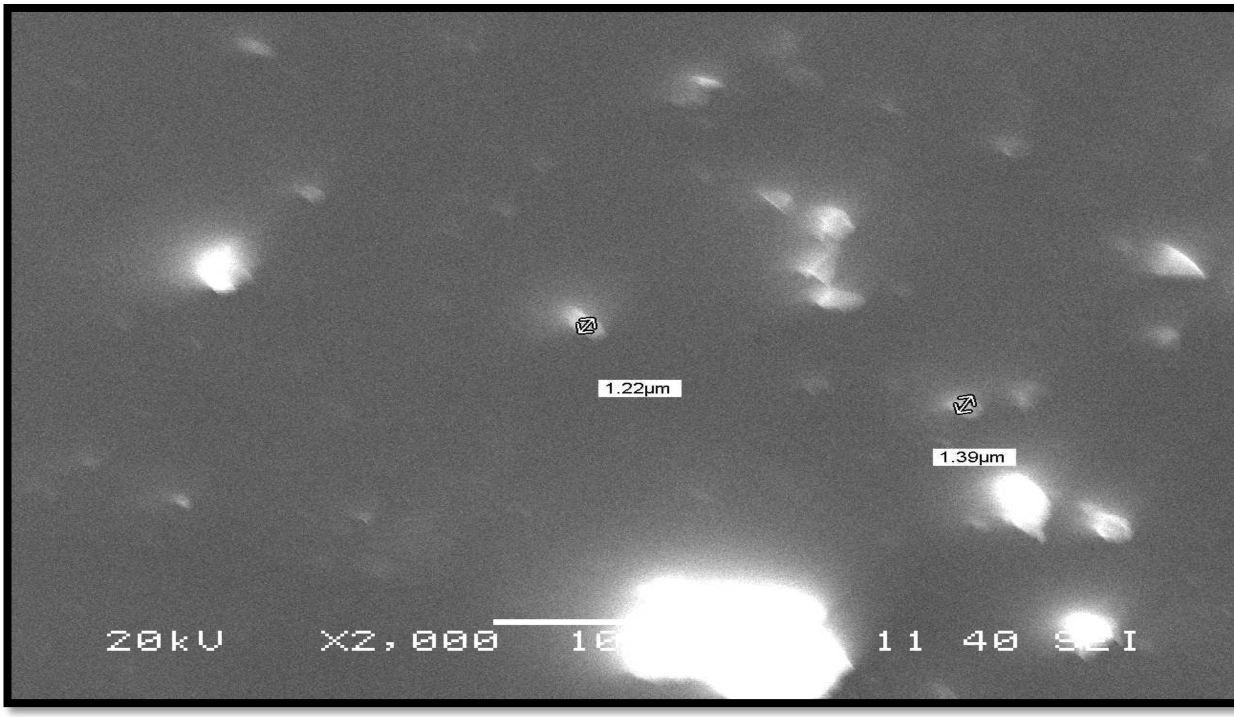

Fig.11: SEM image for 30:1 ratio silver nanoparticles

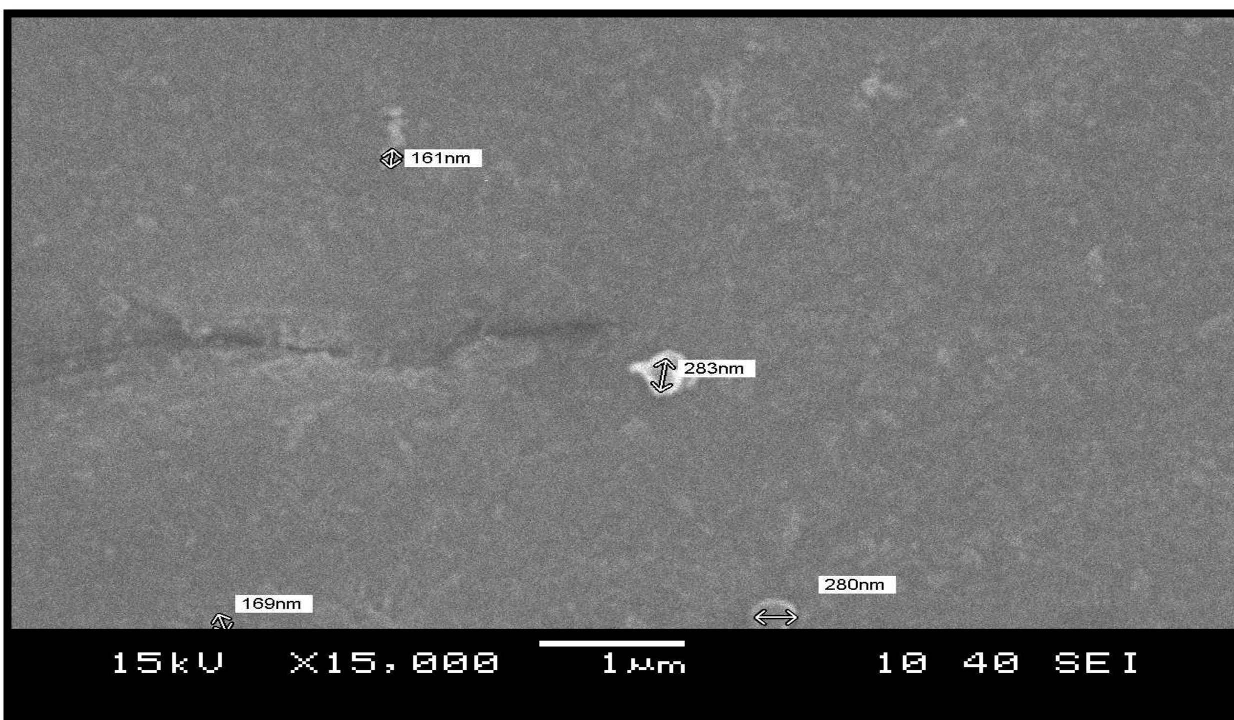

Fig.12: SEM image for 60:1 ratio silver nanoparticles

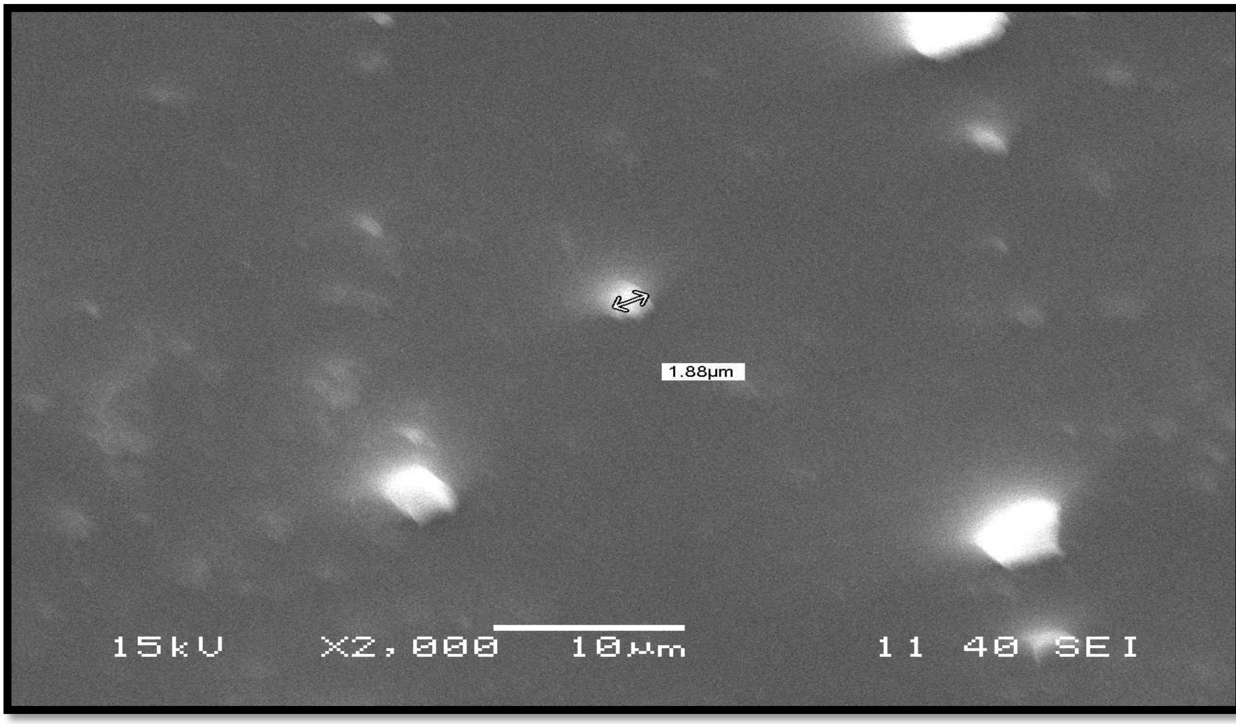

Fig.13: SEM image for 120:1 ratio silver nanoparticles

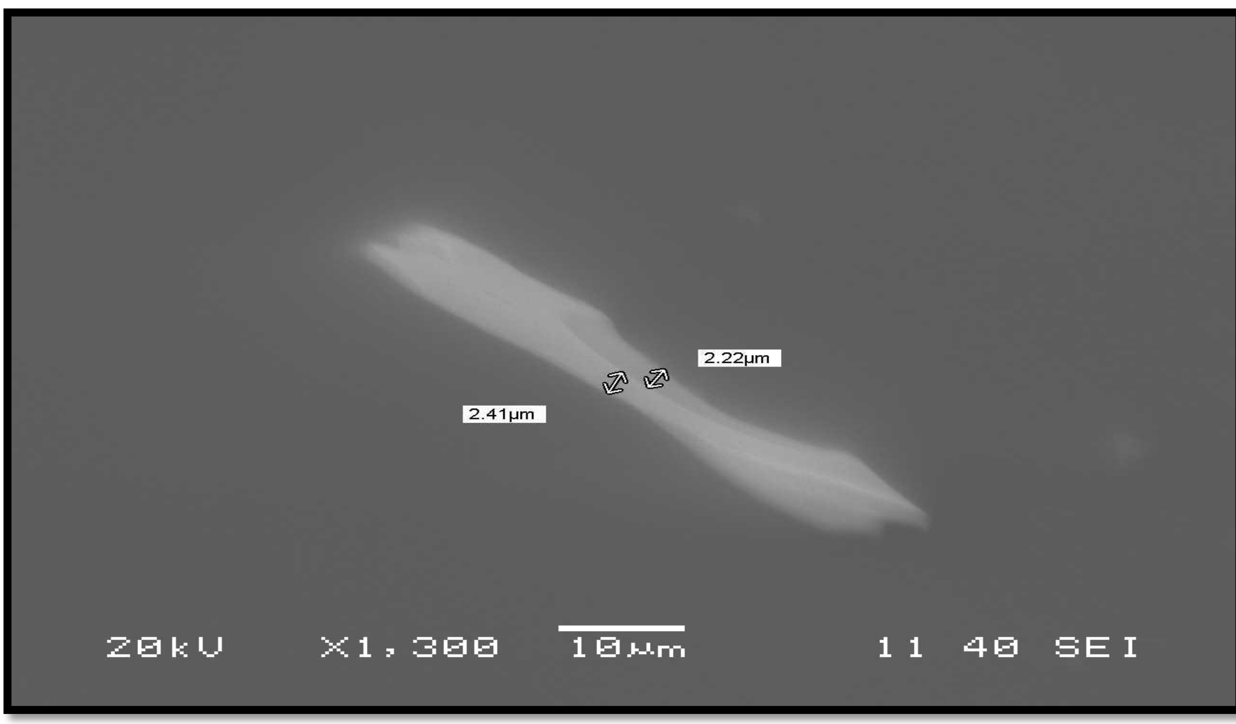

Fig.14: SEM image for 240:1 ratio silver nanoparticles

DLS ANALYSIS:

The particle size distribution (PSD) of synthesized silver nanopaericles of different ratios like 30:1, 60:1, 120:1, and 240:1 are shown in the figures. **(Figure: 15, Figure: 16, Figure: 17 & Figure: 18)**.According to the **figure: 15** the colloidal solution of silver nanoparticles of ratio 30:1 contains particles of different sizes some were with average sizes ranging from 5 nm to 180 nm. But in case of 60:1, the solution contains particles of uniform sizes ranging from 68 nm to 396 nm. The average size of nanoparticles is 160 nm. The particle size in case of 120:1 ratio ranges from 78 nm to 255 nm with mean particle size of 169 nm. Similarly the sizes of nanoparticles in case of 240:1 ratio range from 91 nm to 220 nm with average size of 164 nm. If we compare the above four results we can conclude that the ratios like 60:1, 120:1, 240:1 give uniform distribution of particles but 30:1 ratio does not obey this principle. Among them 60:1 ratio is very appropriate since it gives lowest average size of nanoparticles.

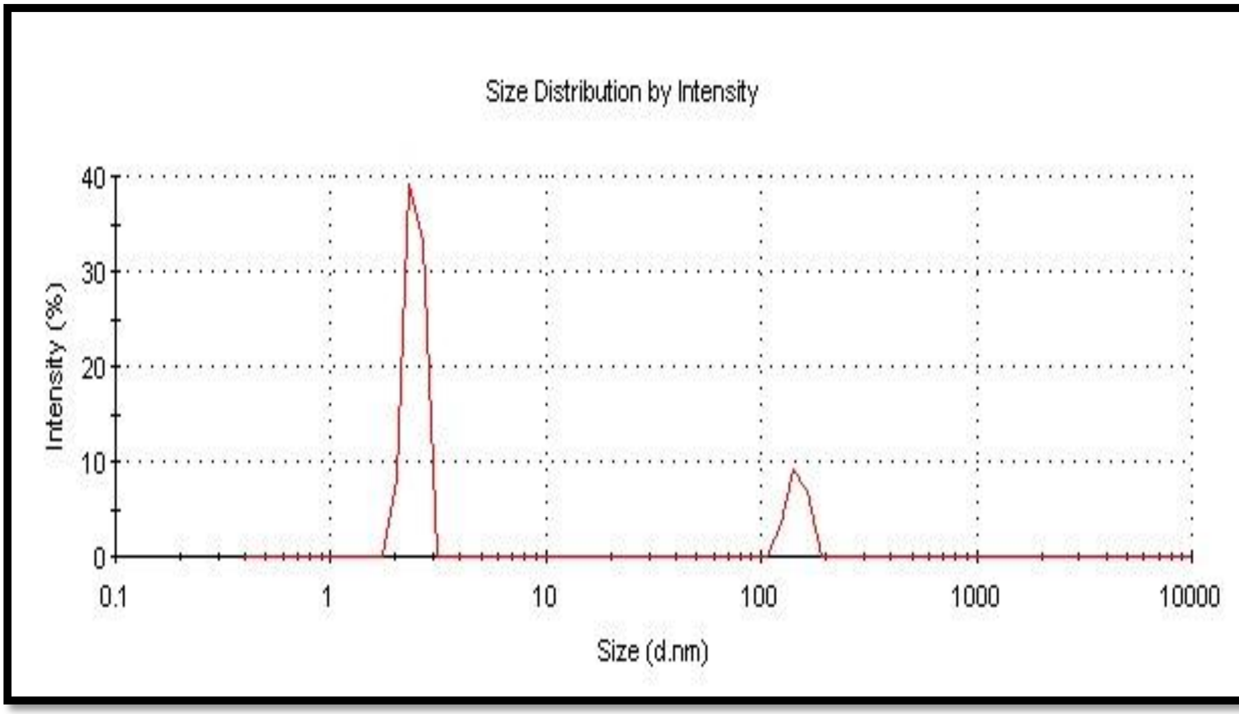

Fig.15: DLS result for 30:1 ratio silver nanoparticles

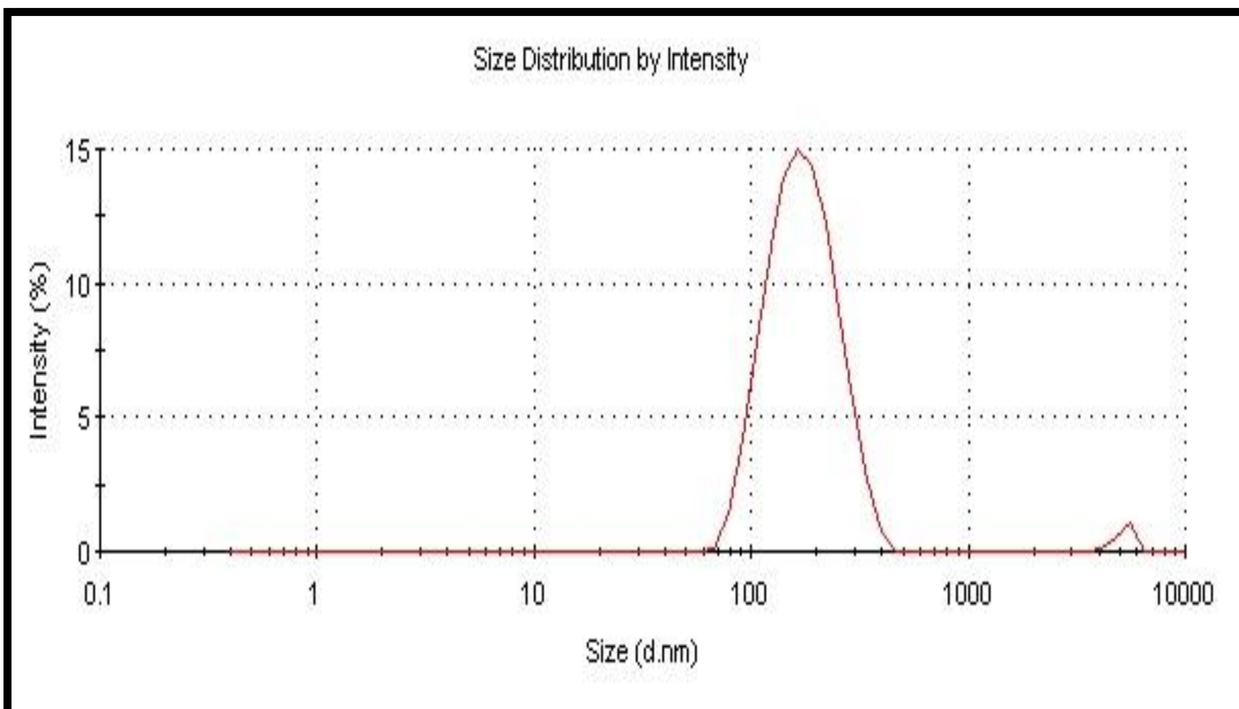

Fig.16: DLS result for 60:1 ratio silver nanoparticles

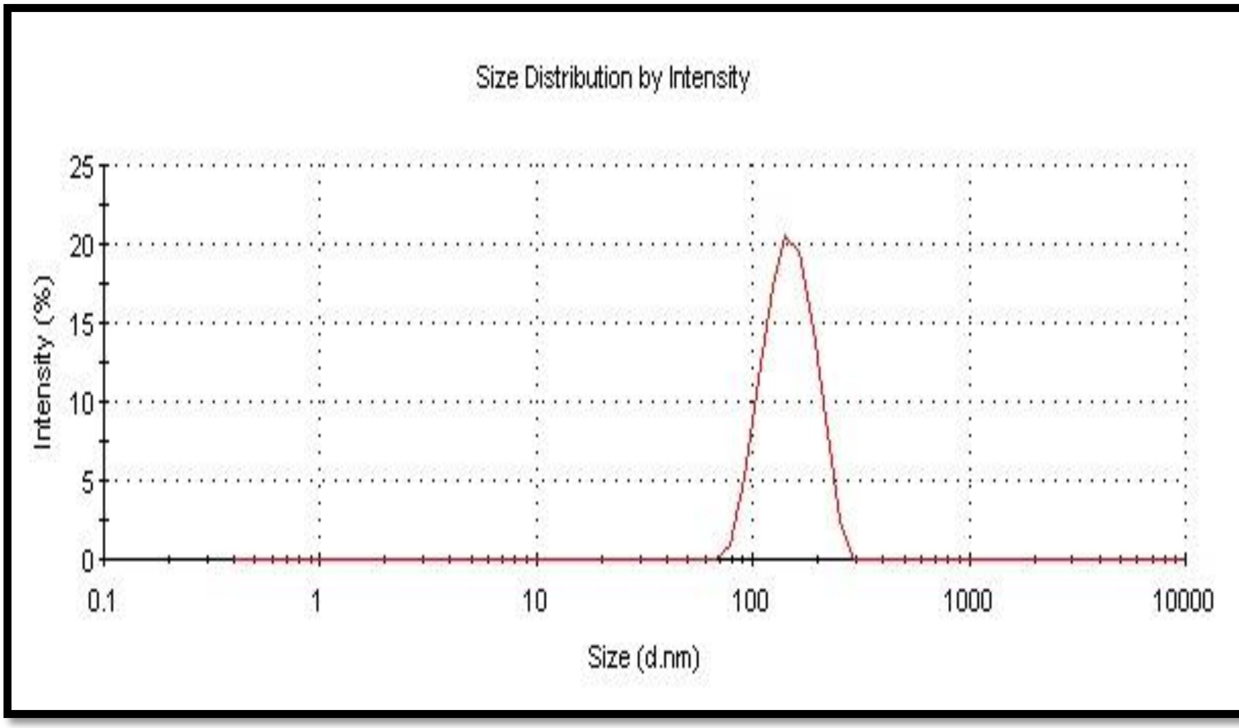

Fig.17: DLS result for 120:1 ratio silver nanoparticles

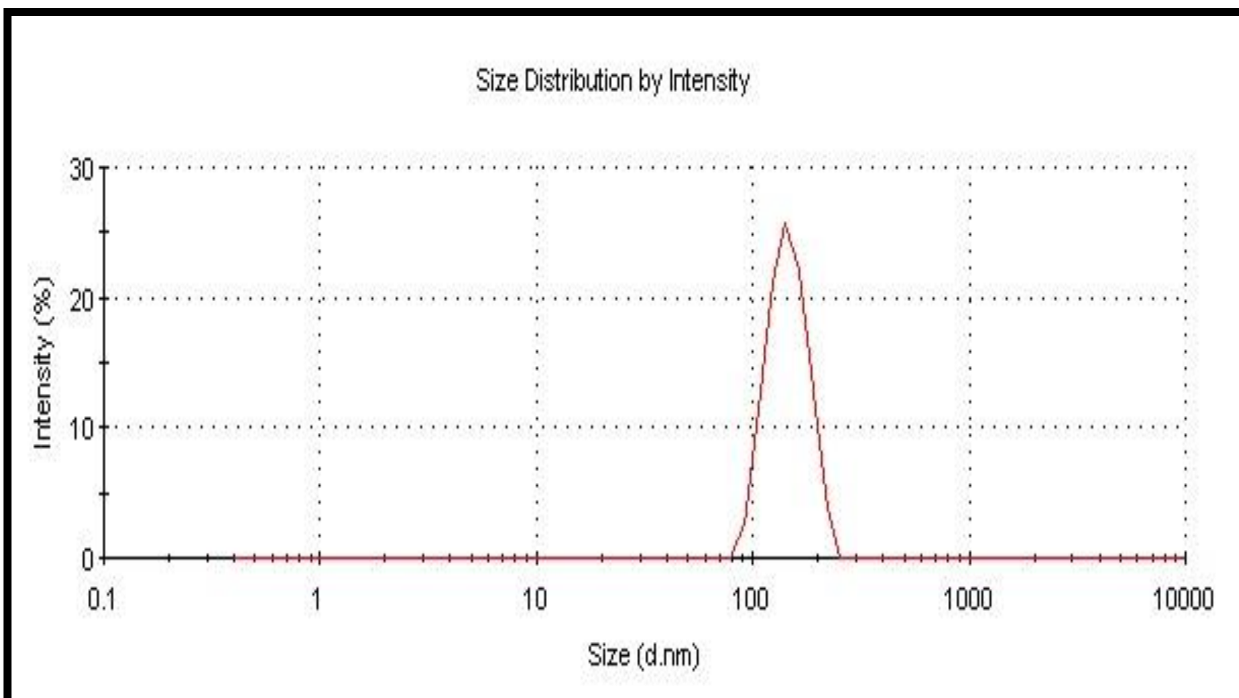

Fig.18: DLS result for 240:1 ratio silver nanoparticles

ZETA POTENTIAL ANALYSIS:

The Zeta potential measurements of silver nanoparticles synthesized with different ratios like 30:1, 60:1, 120:1, and 240:1 are 15.5 mV, 1.92 mV, 6. 12 mV, and 2.45 mV respectively. (**Figure: 19, Figure: 20, Figure: 21 & Figure: 22)**. From the analysis the order of stability of nanoparticles synthesized from different ratios is 30:1> 120:1 > 240:1 > 60:1. Nanoparticles are very small in size for which they are energetically very unstable. Therefore the particles undergo agglomeration/aggregation to stabilize themselves. So there were some potential charges on the surface of the nanoparticles which makes them stable. These charge potential we got from this analysis.

Zeta potential (Surface potential) has direct relation with the stability of a form/structure as mentioned below **(Table: 3)**

Table: 3: A table showing the stability of the NPs according to the potential charge

Zeta potential [mV]	Stability behavior of the colloid
from 0 to ±5	Rapid coagulation or flocculation
from ±10 to ±30	Incipient instability
from ±30 to ±40	Moderate stability
from ±40 to ±60	Good stability
more than ±61	Excellent stability

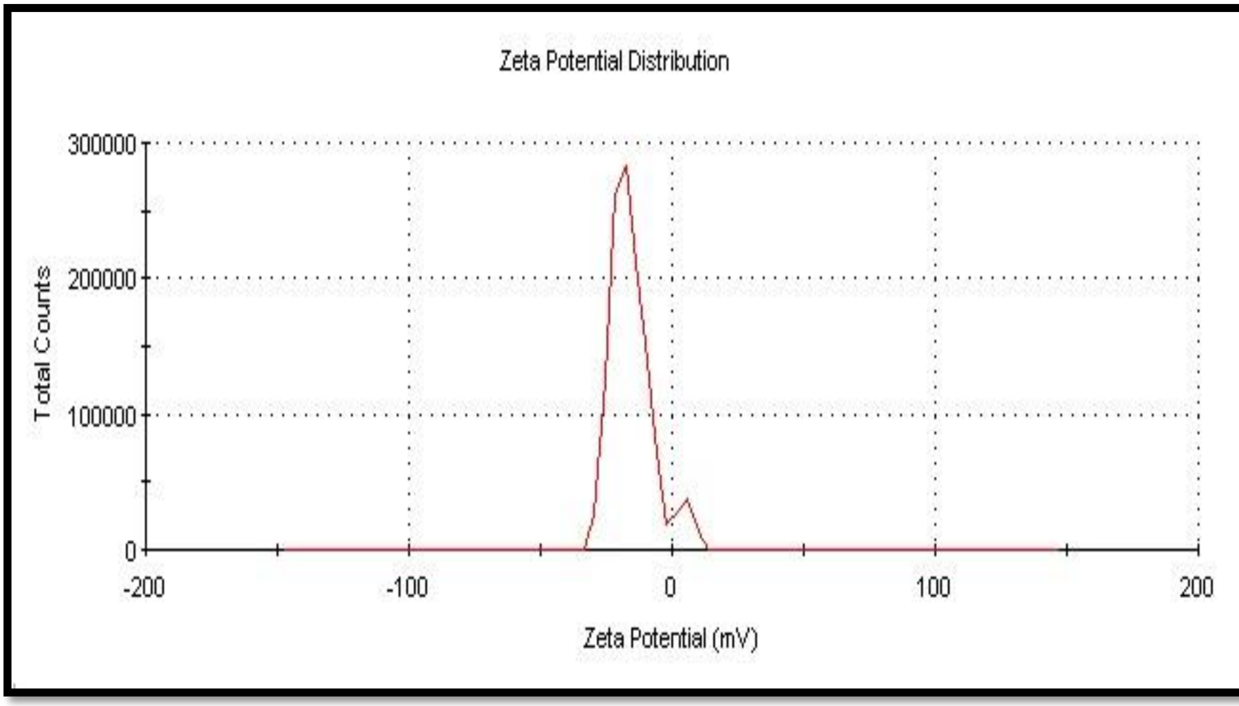

Fig.19: Zeta Analysis result for 30:1 ratio silver nanoparticles

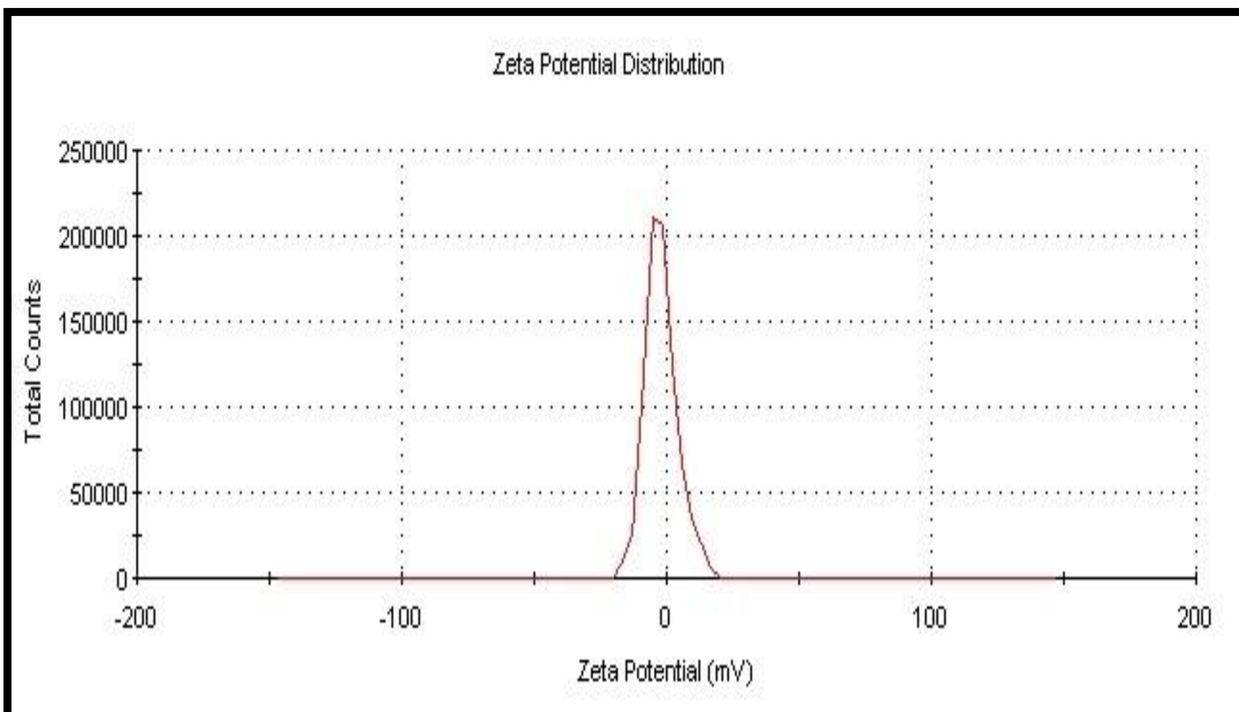

Fig.20: Zeta Analysis result for 60:1 ratio silver nanoparticles

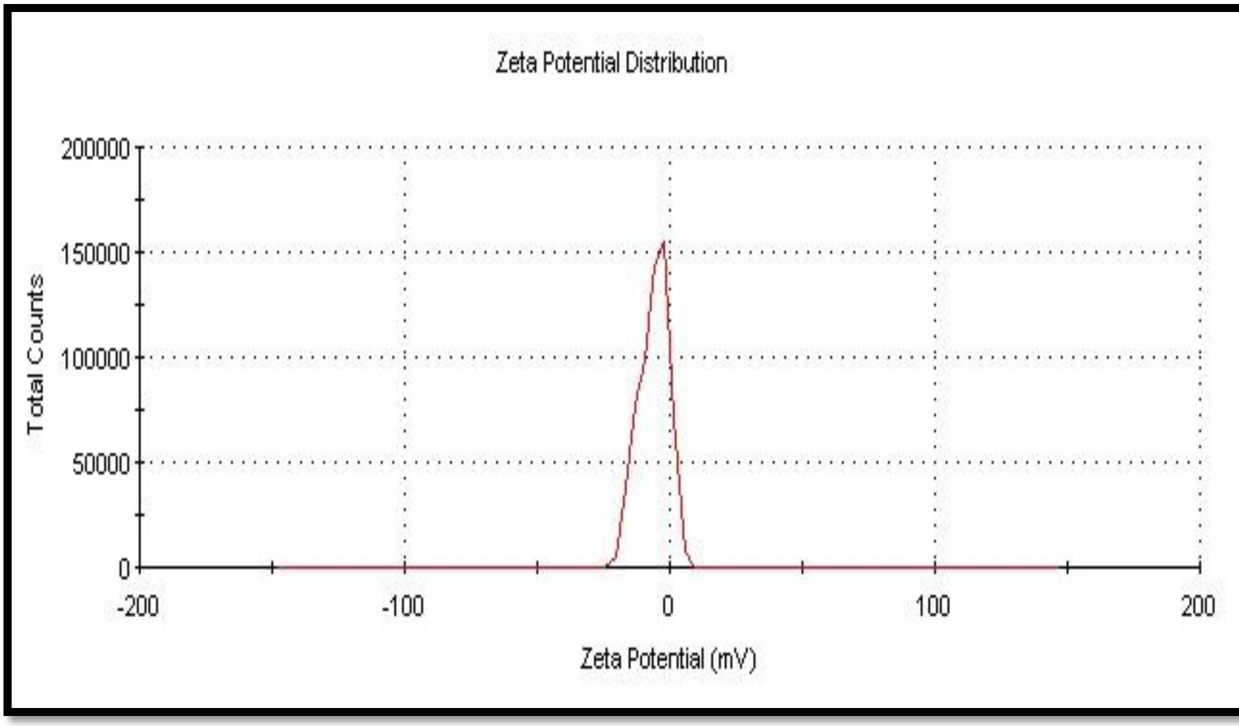

Fig.21: Zeta Analysis result for 120:1 ratio silver nanoparticles

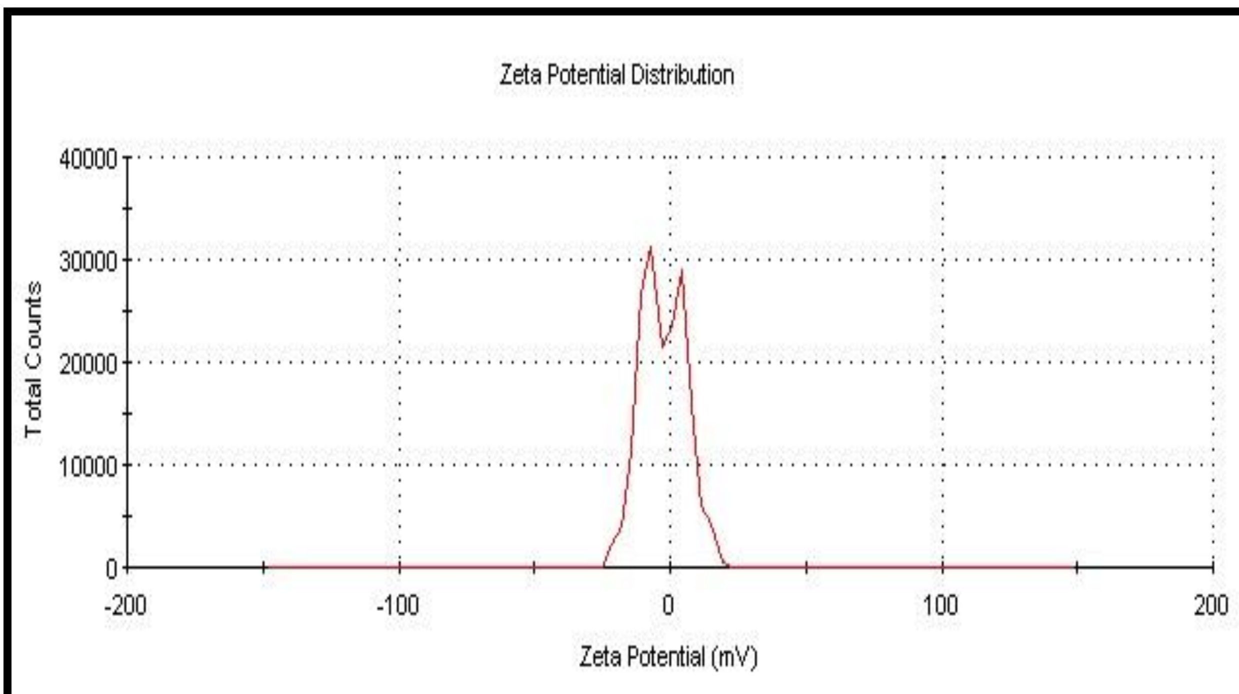

Fig.22: Zeta Analysis result for 240:1 ratio silver nanoparticles

FTIR ANALYSIS:

FTIR measurements were carried out to identify the biomolecules for capping and efficient stabilization of the metal nanoparticles synthesized. The FTIR spectrum of silver nanoparticles **(Figure: 23 & Figure: 24)** in case both of 60:1 and 120:1 ratios showed the band between 3490-3500 cm^{-1} corresponds to O-H stretching H-bonded alcohols and phenols. The peak found around 1500-1550 cm^{-1} showed a stretch for C-H bond, peak around 1450-1500 cm^{-1} showed the bond stretch for N-H. Where as the stretch for Ag-NPs were found around 500-550 cm^{-1}. Therefore the synthesized nanoparticles were surrounded by proteins and metabolites such as terpenoids having functional groups. From the analysis of FTIR studies we confirmed that the carbonyl groups from the amino acid residues and proteins has the stronger ability to bind metal indicating that the proteins could possibly from the metal nanoparticles (i.e.; capping of silver nanoparticles) to prevent agglomeration and thereby stabilize the medium. This suggests that the biological molecules could possibly perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium. Carbonyl groups proved that flavanones or terpenoids absorbed on the surface of metal nanoparticles. Flavanones or terpenoids could be adsorbed on the surface of metal nanoparticles, possibly by interaction through carbonyl groups or π-electrons in the absence of other strong ligating agents in sufficient concentration. The presence of reducing sugars in the solution could be responsible for the reduction of metal ions and formation of the corresponding metal nanoparticles. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acids. These issues can be addressed once the various fractions of the neem leaf extract are separated, identified and individually assayed for reduction of the metal ions. This rather elaborate study is currently underway.

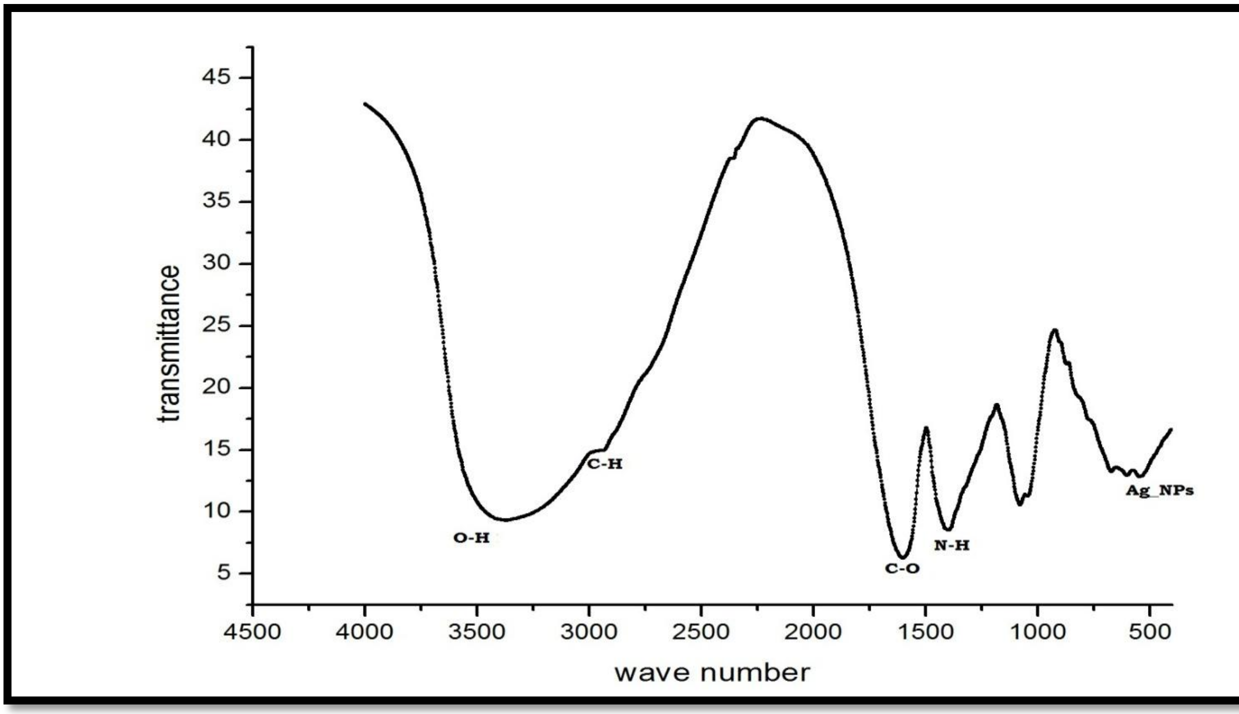

Fig.23: FTIR result for 60:1 ratio silver nanoparticles

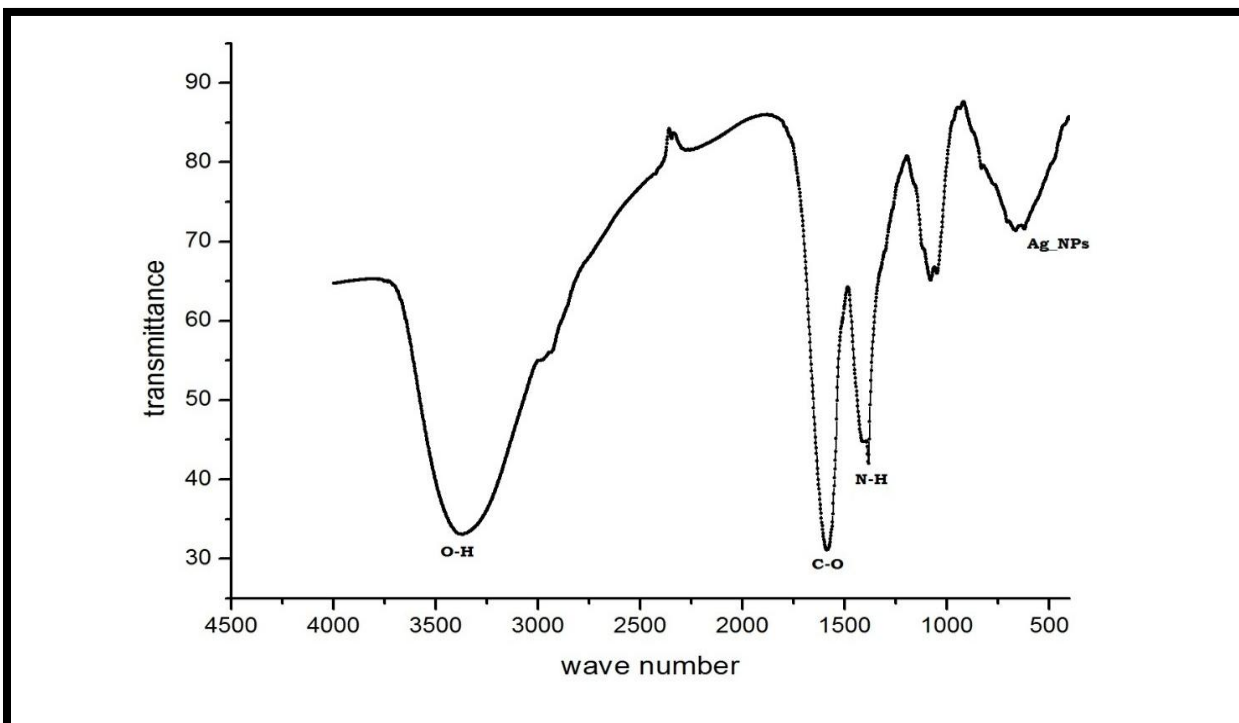

Fig.24: FTIR result for 120:1 ratio silver nanoparticles

XRD ANALYSIS:

XRD spectrum **(Figure: 25)** showed distinct diffraction peaks around 38^{O}, which are indexed by the (100) of the cubic face-centered silver. These sharp Bragg peaks might have resulted due to capping agent stabilizing the nanoparticle. Intense Bragg reflections suggest that strong X-ray scattering centres in the crystalline phase and could be due to capping agents. Independent crystallization of the capping agents was ruled out due to the process of centrifugation and redispersion of the pellet in millipore water after nanoparticles formation as a part of purification process. Therefore, XRD results also suggested that the crystallization of the bio-organic phase occurs on the surface of the silver nanoparticles or vice versa. Generally, the broadening of peaks in the XRD patterns of solids is attributed to particle size effects. Broader peaks signify smaller particle size and reflect the effects due to experimental conditions on the nucleation and growth of the crystal nuclei.

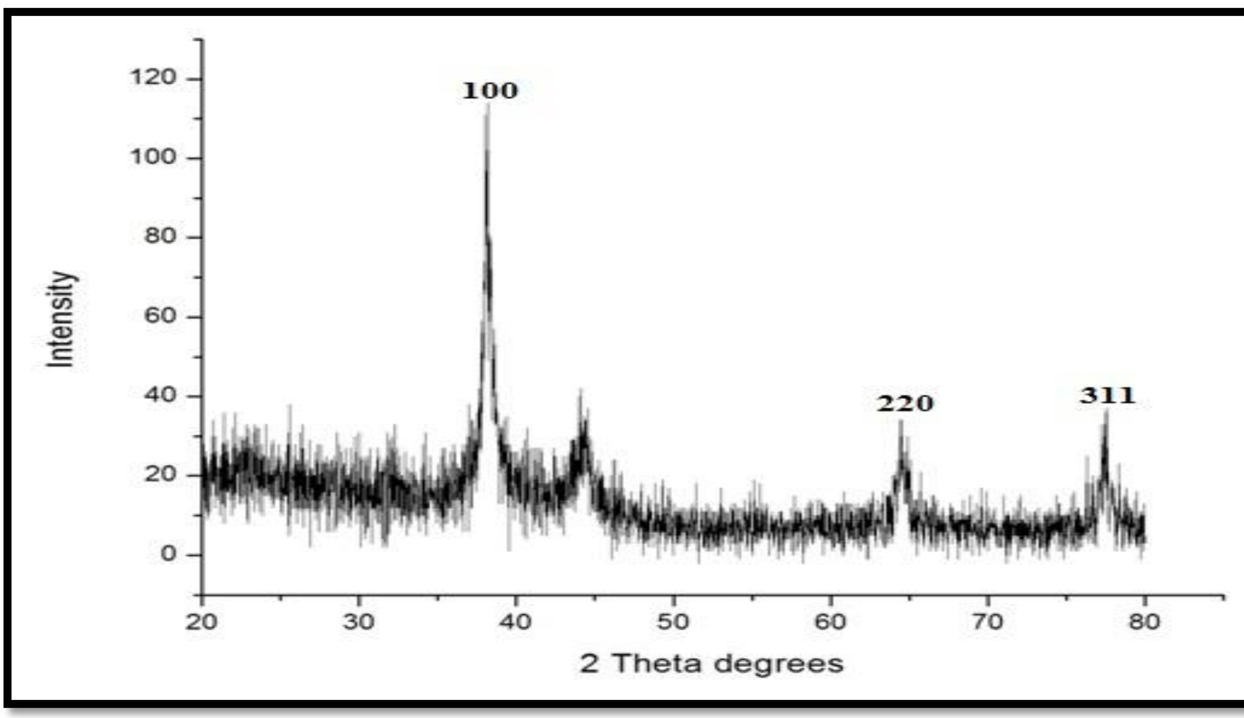

Fig.25: XRD result for 60:1 ratio silver nanoparticles

CONCLUSION

The rapid biological synthesis of silver nanoparticles using *Azadirachta indica* leaves extract provides environmental friendly, simple and efficient route for synthesis of benign nanoparticles. The synthesized nanoparticles were of spherical and sheet shaped and the estimated sizes were 160-180 nm. The size were bigger as the nanoparticles were surrounded by a thin layer of proteins and metabolites such as terpenoids having functional groups of amines, alcohols, ketones, aldehydes, etc., which were found from the characterization using UV-vis spectrophotometer, SEM, DLS, Zeta Analyzer, XRD, and FTIR techniques. All these techniques it was proved that the concentration of plant extract to metal ion ratio plays an important role in the shape determination of the nanoparticles. The higher concentrated nanoparticles had sheet shaped appearance where as the lower concentrations showed spherical shaped. The sizes of the nanoparticles in different concentration were also different which depend on the reduction of metal ions. From the data of DLS it was found that the 30:1 ratio solution had sharp nanoparticles of around 5 nm and some has around 180 nm and the had the potential of around 15.5 mV. From the technological point of view these obtained silver nanoparticles have potential applications in the biomedical field and this simple procedure has several advantages such as cost-effectiveness, compatibility for medical and pharmaceutical applications as well as large scale commercial production.

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